Bacterial attachment and biofilm development

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Thesis submitted for the degree of Doctor of Philosophy

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September 1993

DECLARATION

The work contained in this thesis is the result of original work by myself under the supervision of Dr C.S. Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree

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September 1993

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ABBREVIATIONS

AMPS	Ammonium persulphate
BATH	Bacterial adhesion to hexadecane
CFU	Colony forming units
cm	Centimetre
d	Dilution rate
DNA	Deoxyribonucleic acid
DOS	Direction of spreading
EIC	Electrostatic interaction chromatography
EPS	Extracellular (Exo) polysaccharide
g	Gram
g	gravitational force
HIC	Hydrophobic interaction chromatography
hr	hour
KDa	Kilodalton
LPS	Lipopolysaccharide
M	Molar
mA	Milliamp
MATH	Microbial adhesion to hydrocarbons
μ	specific growth rate
μg	Microgram
μm	Micometre
μmRa	Roughness average
mg	Milligram
min	Minute
mM	Millimolar
mm	Millimetre
ng	Nanogram

nm	Nanometre
^{o}C	Degrees celsius
OD	Optical density
OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
%	Percentage
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SAT	Salt aggregation technique
SDS	Sodium dodecyl sulphate
sec	Second
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylaminomethane
TLC	Thin layer chromatography
Tris	Tris-hydroxymethylaminomethane
v/v	Volume by volume
w/v	Weight by volume

ACKNOWLEDGEMENTS

I would like to thank those who have helped me throughout this project. Thanks to Dr Crawford Dow for his encouragement, advice and for his help preparing this thesis. Thanks also to Dr John Holah for the introduction to the food industry and for giving me the opportunity to put my biofilm experience into practice. I would also like to thank Sian, Sue, Ifti, Pete, Uthaya and everyone else in the lab, as well as the many friends I made at Warwick for making my time at Warwick so enjoyable.

Finally, thanks to Jasp for his never-ending support and encouragement, and particularly for the extensive computing support !!

SUMMARY

Initial work involved the isolation and characterization of bacteria found on the surfaces in a range of food processing environments. The attachment characteristics of three species of bacteria (*S.liquefaciens, S.cohnii* and *P.fragi*) isolated from the same production surface were examined. Mixed culture biofilm development was also modelled using these three organisms. The species were found to have differing attachment abilities and therefore cell surface characteristics such as outer membrane protein and lipopolysaccharide profiles, exopolysaccharide production, and cell surface hydrophobicity and charge were examined to explain these differences. All of these cell surface characteristics were subject to considerable variation in response to the environmental conditions. The outer membrane protein and lipopolysaccharide profiles did not show any relationship to attachment levels, and similarly exopolysaccharide production did not relate to the levels of attachment observed, although exopolysaccharide production was found to be particularly associated with attached cells. The attachment of *S.cohnii* and *P.fragi* correlated to cell surface hydrophobicity, whilst the attachment of *S.liquefaciens* was dependent upon cell surface charge.

CHAPTER ONE

1. INTRODUCTION

Microbes readily attach to surfaces in a wide variety of environments. This thesis examines the attachment and development of biofilms on stainless steel in order to elucidate the cell surface characteristics involved in the attachment of organisms commonly found in the food processing environment.

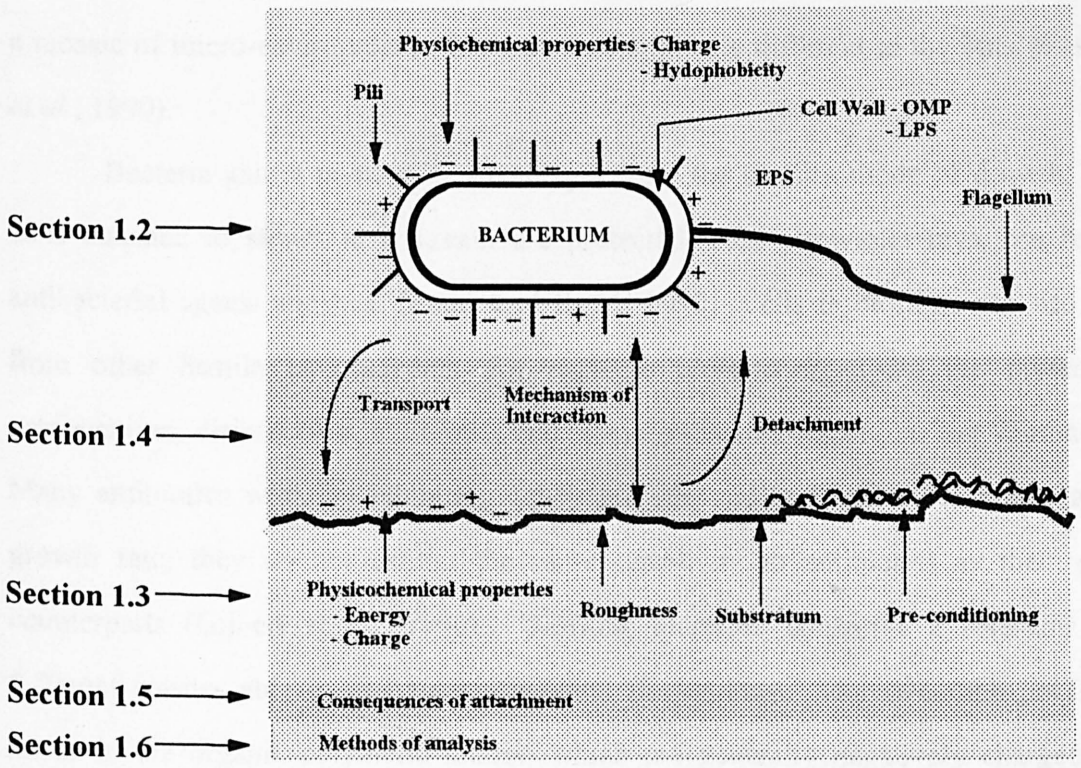
The introduction first reviews the environments and industries affected by microbial attachment and biofilm development. The layout of the remaining sections is illustrated in Figure 1.1. The second section describes the cell surface characteristics that may play a role in attachment (section 1.2). The third section evaluates the influence of substratum characteristics in the adhesion process (section 1.3). The fourth section examines the processes thought to be involved in attachment and physico-chemical theories developed to predict adhesion (section 1.4). The fifth part reviews the various reported consequences of attachment and biofilm growth for the cell (section 1.5). The penultimate section summarises the methods and techniques used to analyse attachment and biofilm development and the final section describes the aims of the work and the approaches taken.

1.1 Introduction to biofilms

According to McFeters *et al.* (1984) a biofilm consists of micro organisms and extracellular substances in association with a substratum. Characklis (1989) defines a biofilm as consisting of cells immobilized on a substratum and often embedded in a matrix of microbially produced extracellular polymers. This matrix consists largely of water (98-99% according to Christensen and Characklis, 1990) and may contain various extracellular polymers, commonly polysaccharides and glycoproteins. The bacteria are not uniformly distributed throughout the biofilm as the biofilm consists of microcolonies with channels in between resulting in a rather heterogeneous structure (Geesey *et al.*, 1992).

Figure 1.1

Diagram illustrating the structure of the introduction



Microbial cells attach firmly to almost any surface in a wide variety of natural environments, for example microbes are found on almost all surfaces in marine and freshwater systems with algal biofilms developing on any illuminated surface which is either submerged in water or in a humid environment (Leadbetter and Callow, 1992). Transmission and scanning electron microscopic studies have shown that immobilized cells grow, reproduce and synthesize extracellular polymers which frequently extend from the cell forming an extracellular matrix (Characklis, 1990). The adsorptive nature of this matrix means that particulates may be trapped and inorganic components may bind to the matrix. Sessile or attached microbial populations exist on surfaces in flowing systems and are often present in higher numbers than the planktonic population, particularly in oligotrophic systems (Blenkinsopp and Costerton, 1991). Biofilms range from monolayers of cells to the

complex mixtures found in algal mats which may be up to 300-400 mm thick. Biofilms are ubiquitous in nature and the organisms often exist as members of complex consortia, rather than pure cultures. Their localised metabolic activity can create diffusion gradients of nutrients, fermentation by products and other products associated with corrosion resulting in a mosaic of micro-environments that may be completely different to the bulk phase (Keevil *et al.*, 1990).

Bacteria gain a number of advantages from the biofilm mode of growth; microbial cells attached to stones in a stream are protected from ultraviolet rays, are resistant to antibacterial agents and heat (Frank and Koffi, 1990). Cells in biofilms are also protected from other hostile environments, for example biofilm organisms are protected from preservatives, disinfectants, antibiotics and biocides (Mattila-Sandholm and Wirtanen, 1992). Many antibiotics work by inhibiting active growth, and as biofilm cells have a different growth rate, they do not exhibit the same sensitivity to antibiotics as their planktonic counterparts (Gilbert *et al.*, 1990). Biofilms therefore constitute a reservoir of many different species able to resist environmental fluctuations. Moreover, the polysaccharide fibres in the organic polymeric matrix, which are generally negatively charged, trap the organic and mineral molecules and particles in the bulk phase (Carpentier and Cerf, 1993).

1.1.1 Relevance of biofilms

Biofilm formation causes problems in many branches of industry and is generally referred to as biofouling. This is a natural phenomenon that occurs wherever suitable conditions exist for biofilm formation. In industry the presence of biofilms may cause problems for the particular operation or process, in addition the quality of the product may be adversely affected (Bott, 1992a and b). Examples of processes affected by biofilms, and those utilizing biofilms are listed in Table 1.1.

Biofilms may serve beneficial purposes in the natural environment, for example benthal communities are responsible for the removal of dissolved and particulate contaminants from natural streams (Blenkinsopp and Lock, 1990; Jones and Lock, 1989); they often affect water quality by influencing dissolved oxygen and by serving as a sink of

many toxic and/or hazardous materials and may play a role in the cycling of nutrients (Characklis, 1990). The organic and nutrient trapping properties are successfully used in waste water treatment to reduce organic content (Capdeville *et al.*, 1992; Mendez and Lema, 1992; Christensen *et al.*, 1989) and for waste gas treatment to reduce air pollution (Reij *et al.*, 1992). In addition biofilms in treatment plants have been used to remove heavy metals from solution as the negatively charged extracellular polysaccharides have a high affinity for metallic cations. The oil industry exploits the plug forming ability of biofilms to enable secondary oil recovery to occur (Blenkinsopp and Costerton, 1991).

Problems associated with biofilms can cause significant financial losses to industry. The algal and bacterial fouling of ship hulls results in greatly increased fuel consumption and necessitates expensive periodic scraping to remove growth and the application of antifouling paints (Heath *et al.*, 1992). The output of power station heat exchangers which may have up to 100 km of pipes is significantly affected by the biofilms that develop on the river water cooled side of the heat exchangers (Bott, 1992). Any system that involves water flowing through pipelines e.g. drinking water distribution systems suffers from reduced flow rates due to biofilm growth. Health risks also exist in the water treatment industry as large amounts of biofilm may slough off the pipe walls causing the concentration of cells in the planktonic phase to rise above levels considered safe for human consumption. Biofilms in water distribution systems may act as a reservoir of potentially pathogenic organisms as the biofilm organisms are resistant to even high levels of chlorine (Block, 1992). Herson *et al.* (1987) found that *Enterobacter cloacae* in drinking water was protected from the action of chlorine when attached to surfaces. The cells are protected by a network of exopolymers, which inactivates the chlorine thus forming a stable ecosystem that is difficult to remove. Work by Vess *et al.* (1993) showed that common water bacteria can attach and colonize the interior of PVC pipes and develop significant resistance to the action of certain germicides. The primary mode of dispersion of *Legionella pneumophila* is in aerosols and frequently occurs in cooling water towers or contaminated drinking water. Once attached to surfaces, *L.pneumophila* is far more resistant to biocides (Wright *et al.*, 1991).

Biofilms consisting of anaerobic sulphate reducing bacteria and other sulphate

metabolizing organisms are associated with the corrosion of metals in diverse industrial installations (Costerton and Lashen, 1984; Benbouzid-Rollet *et al.*, 1991). Industrial biofilms may also pose a health risk. In the oil industry, stringent precautions are taken to avoid inhalation of toxic levels of hydrogen sulphide generated by bacteria within biofilms in pipelines and in oil producing formations themselves (Blenkinsopp and Costerton, 1991).

Bacteria such as *Pseudomonas aeruginosa, Escherichia coli* and certain Gram positive organisms readily colonize medical implants such as catheters resulting in biofilms that are extremely recalcitrant (Fletcher, 1992; Busscher *et al.*, 1992). Stickler and Hewett (1991) found that mixed bacterial biofilms on silicone were particularly resistant to antiseptics. Another infection associated with the attachment of bacteria is the formation of dental plaque due to the attachment of oral bacteria to the tooth surface resulting in carious lesions if streptococci and lactobacilli are involved (Busscher *et al.*, 1992).

1.1.2 Biofilms in the food industry

The cleanliness of surfaces, equipment and the environment in the food industry affects the quality of the products processed. From a quality point of view, the product should be free of spoilage organisms and foreign bodies and from a safety point of view pathogens should be absent. Contamination of the product may arise from four main sources; the constituent raw materials, surfaces, people (and other animals) and the air. The quality of the raw materials is controlled by positive release systems for materials after sampling, quality control and assurance procedures and supplier auditing. The raw material route of contamination is the only non factory environment route of contamination. Food may also pick up contamination as it is moved across product contact surfaces or if it is touched by food handlers or pests. The air can also act as a source of contamination, and in addition may act as a transport medium carrying contamination from non food contact surface to product contact surface (Holah and Kearney, 1992).

Table 1.1

Effects and relevance of biofilms (adapted from Characklis, 1981 and 1990)

Effect	Examples
Heat transfer reduction	Biofilm formation on condenser tubes and cooling towers result in energy losses
Increase in fluid frictional resistance	Biofilm development in water and waste water conduits causes increased power consumption
Increase in fluid frictional resistance	Biofilm formation on ship hulls results in increased fuel consumption
Reduced performance	Biofilm formation on remote sensors, submarine periscopes etc. reduces effectiveness
Reduced performance	Biofilm accumulation reduces the effectiveness of ion exchange and membrane processes used in water treatment
Reduction of product quality	Pulp and paper industry are detrimentally affected by biofilm formation
Reduction of product quality	Biofilms in food processing environments may be a reservoir of spoilage organisms
Health risks	Biofilm formation and detachment in drinking water systems changes water quality
Health risks	Detachment of microorganisms from cooling towers may release pathogens such as *Legionella* in aerosols
Health risks	Biofilm accumulation on teeth and gums, urinary and intestinal tracts responsible for disease
Health risks	Detachment of pathogens from biofilms in food processing environments may lead to food poisoning
Increased product yield	Immobilized microbes are used in biotechnological industries to give improved productivity and process stability
Increased product yield	Extraction of minerals from ores can be mediated by biofilm organisms
Maintenance of water quality	Benthal biofilm stream activity purifies the water
Reduction of water pollutant load	Extraction and oxidation of organic and inorganic compounds from waste water can be mediated by immobilized organisms

Food processing environments provide a variety of conditions which might be expected to favour the formation of biofilms e.g. flowing water, suitable attachment surfaces, ample nutrients (although possibly sporadic) and raw materials or the environment supplying the inocula. The time available for biofilm development is usually relatively short as some production lines may operate for a little as an hour, whilst others may run for several days. Consequently the biofilm in food processing environments is more often associated with the attachment and development of microcolonies on the surface rather than thick films that have developed over considerable periods of time. Biofilm development in food processing environments can have detrimental effects on the microbial status of the food product (Holah and Kearney, 1992). Biofilms may harbour a variety of organisms, including pathogens, that can contaminate the product through direct contact or indirectly via vectors such as people, pests, air movement and cleaning systems.

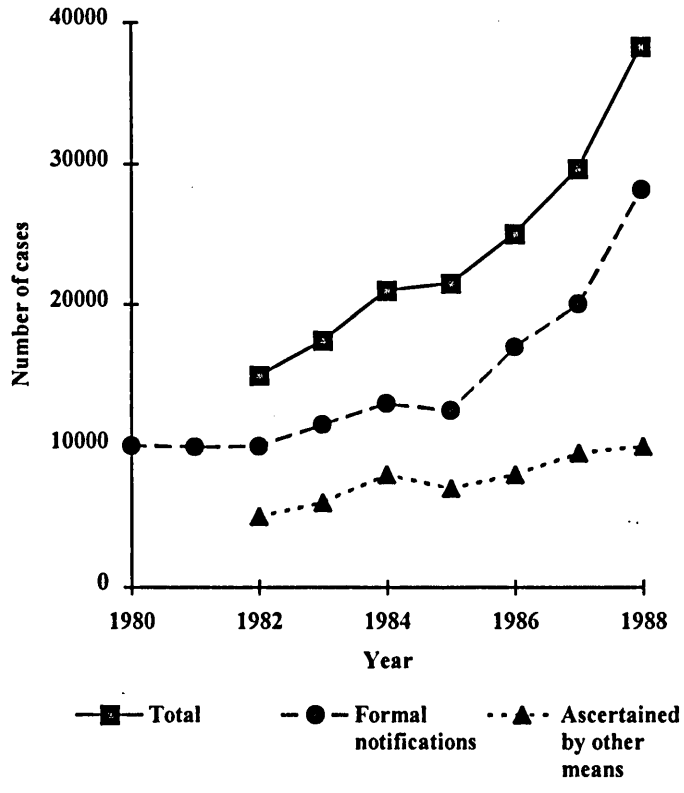
Undoubtably, improved technology in the production, manufacture and distribution of food has led to considerable improvements in hygiene, but at the same time the increases in the scale of production and the scope of distribution open the possibility of larger and more spread incidents. The incidence in food poisoning has increased significantly over recent years (Figure 1.2), therefore demonstrating the requirement for further improvements in hygiene.

Cleaning and disinfection (together known as sanitation) are undertaken to remove all the undesirable material (food residues, micro organisms, foreign bodies and cleaning chemicals) from the surfaces to a level such that the residues remaining are of minimal risk to the safety and quality of the product (Holah, 1992). Sanitation is the major control of the surface route of contamination. When undertaken correctly, cleaning and disinfection regimes are a cost effective way of reducing the risk of microbial and foreign body contamination. This is becoming increasingly pertinent due to the intrinsic demands for higher standards of hygiene required for the production of short shelf life chilled foods and preservative free products. The cleaning and disinfection regime comprises several stages;

Figure 1.2

Notifications of food poisoning in England and Wales between 1980 and 1988

(Source OPCS)



(i) The equipment is dismantled as far as is practical to aid access to the equipment and to facilitate cleaning.

(ii) Gross debris is removed by brushing or scraping and followed by a low pressure cold water rinse to remove loosely adhered debris.

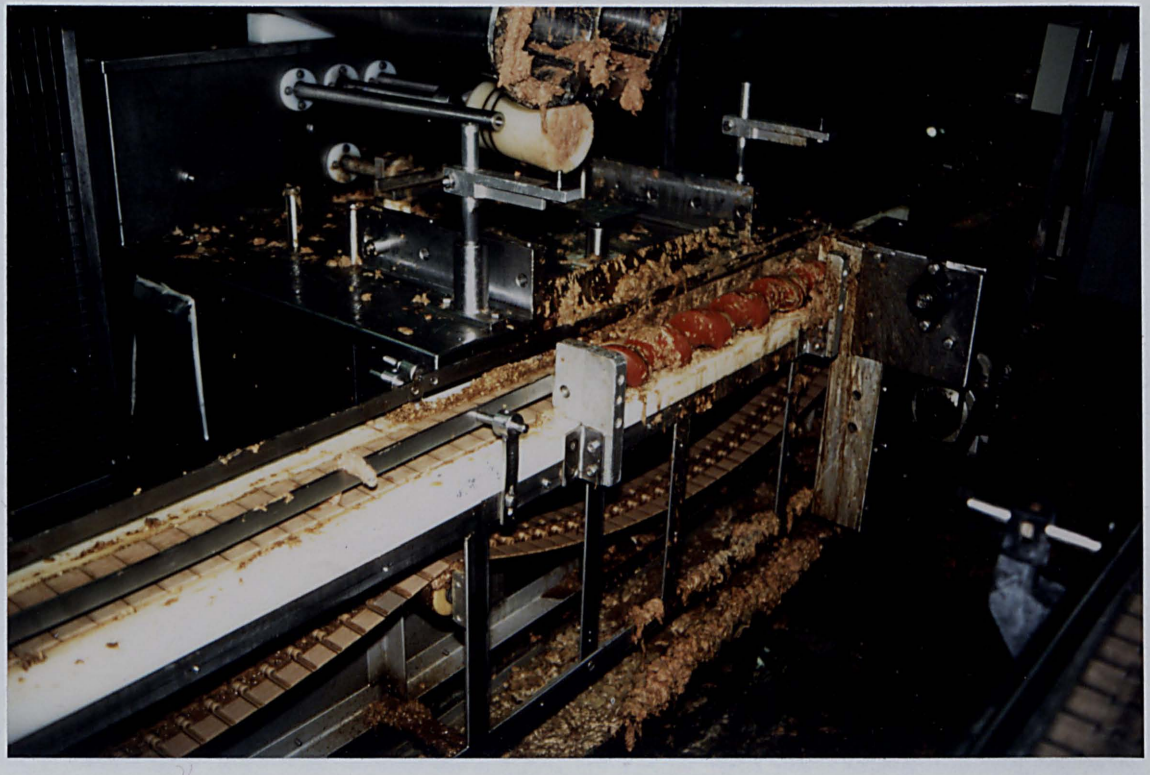
(iii) The equipment is cleaned so that the energy input is sufficient to reduce the interactions between soil particles and the soil and the surface. This energy input comprises a combination of the following; mechanical (kinetic) energy, chemical energy, temperature (thermal energy) and time. The cleaning step is responsible not only for the removal of soil but also the majority of the micro-organisms present. However, detergents are specifically designed to remove a particular soil type rather than attached micro-organisms and therefore

the disinfection stage is required to reduce the surface population further.

(iv) Disinfectants are used to reduce the concentration of organisms remaining on the surface and to prevent bacterial growth on the surfaces. Disinfectant residues are generally rinsed away before production.

Figure 1.3

Typical food processing environment illustrating the range of surface material types present



Biofilms protect micro-organisms from being washed away in the product flow, from cleaning and disinfection and in sites that dry out, from desiccation (Jones, 1993). Various workers have shown that biofilm organisms are more resistant to antibacterial agents (See Keevil and Mackerness, 1990 for a review of biocide treatment of biofilms). Gilbert *et al.* (1990) and Anwar *et al.* (1990) demonstrated that biofilm organisms were more resistant and suggested that this was due to the differences in growth rates between attached and planktonic cells. Biofilm organisms may also be more resistant to steam and formalin (Mattila-Sandholm and Wirtanen, 1992). Holah *et al.* (1990) found that attached organisms were 10-100 times more resistant to disinfectants commonly used in the food industry. Similarly, Le Chevalier *et al.* (1988) demonstrated that biofilm organisms were 150-3000 times more resistant to hypochlorous than unattached cells. Frank (1990) found that *Listeria* species grow in the food processing environment within multi-species biofilms and Frank and Koffi (1990) found that *Listeria monocytogenes* in a biofilm was resistant to all the disinfectants tested and also to heat treatment. There are two ways in which biofilms could result in contamination of the product. Firstly, persistent contamination may be due to a failure of the cleaning regime to remove the biofilms, which can quickly regenerate to act as a source of contamination. Swabs of the main product contact surfaces may fail to detect these hidden reservoirs of infection. Secondly, the biofilm may be the vehicle for spreading contamination from one piece of equipment to another. The cleaning regime often generates aerosols of bacteria and debris, and these aerosolized biofilm fragments may be better protected and survive longer.

Biofilms have been found on a wide variety of both product contact and non product surfaces. Microbial attachment to heat exchanger plates in cheese and liquid milk factories is a well known source of bacterial contamination of dairy products (Bouman *et al.*, 1984) and Zoltai *et al.* (1981) used scanning electron microscopy to demonstrate the adhesion of bacteria to the inside of a milk storage tank. Lewis and Gilmour (1987) also investigated the adhesion of the milk flora to transfer pipes made of rubber and stainless steel. Holah *et al.* (1989) using stainless steel plates or coupons and direct epifluorescent microscopy found that microcolonies rapidly developed on an egg glaze and buttermilk line, and multilayered

biofilms formed on the surfaces of a baked bean production line. Later Holah and Kearney (1992) examined a wider variety of production environments and found cell concentrations up to 10^7 cells cm^{-2}. Flumes, conveyors and slicers have all been shown to be a source of product contamination (Jones, 1993). The recontamination of heat processed foods such as blanched vegetables and cooked meats has been shown to be largely due to micro organisms picked up from the equipment surfaces (Jones, 1993). Environmental surfaces can also harbour bacteria that may be transferred to product by people, pests or cleaning systems. Water is often liberally used in many operations so that static surfaces such as the floors may receive intermittent but regular flows of dilute nutrients and consequently the microbial loading in such environments can be extremely high.

Consequently surface biofilms (product contact surfaces and environmental surfaces) in food processing environments are a significant source of spoilage and / or pathogenic organisms.

Cleaning chemicals have been specifically designed to remove particular types of soil rather than micro organisms. Understanding the attachment of organisms to surfaces in the food environment is the first step in being able to reduce or eliminate this problem.

1.2 Bacterial cell envelope and structures

1.2.1 Bacterial cell surfaces and adhesion

The physicochemical models of adhesion described in section 1.4 treat bacterial cells as inanimate colloidal particles but allow predictions of the forces involved and a thermodynamic measure of the free energy of adhesion. These models could be improved or modified if the relationship between the bacterial cell surface characteristics and the resulting adhesion were examined. An alternative approach would be to examine the bacterial cell surface components and their role in adhesion.

1.2.2 Bacterial cell envelope components

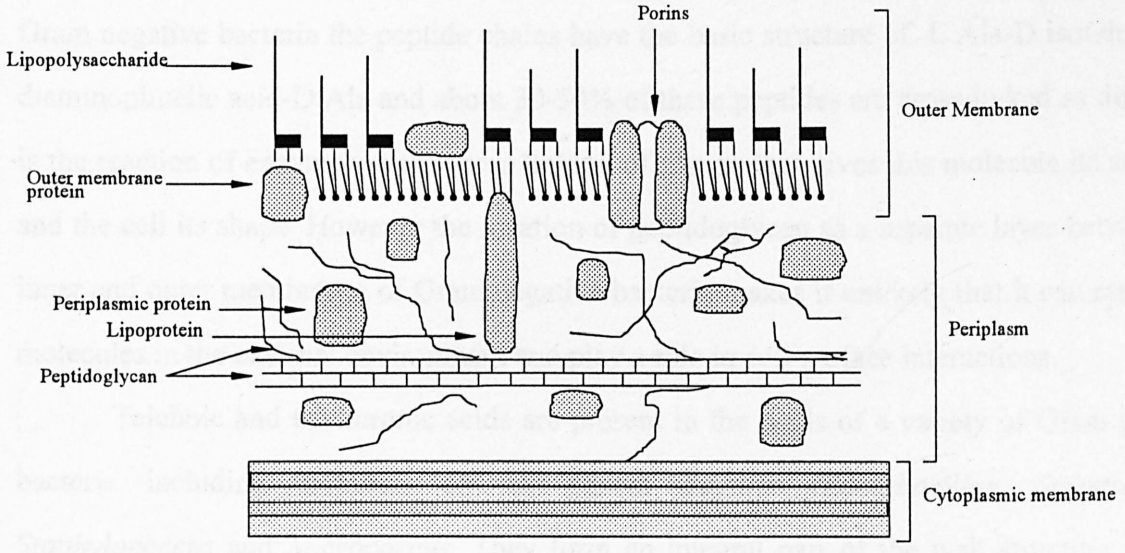
Electron micrographs of thin sections of Gram negative bacteria show the cell envelope to be composed of several layers which are approximately 15-30 nm thick and represents 15-30% of the dry weight of the cell. It consists of two membranes separated by a periplasmic space, which contains a thin dense layer of peptidoglycan overlying the inner membrane. To the exterior of the peptidoglycan is an outer membrane with a unit membrane appearance consisting of proteins, lipoproteins and protein lipopolysaccharide complexes (Figure 1.4). In contrast Gram positive bacteria have a single internal membrane covered with a wall containing peptidoglycan as its main structural component.

1.2.2.1 Plasma membrane

The plasma membrane in both Gram positive and negative organisms is approximately 8 nm thick, represents 2-10% of the dry weight of the cell and consists mainly of phospholipids and proteins. It functions as a selectively permeable barrier preventing the passive movement of polar solutes through the membrane. Active transport mechanisms involving trans-membrane proteins transport nutrients and into and products out of the cell.

Figure 1.4

Schematic diagram of the Gram negative cell wall



1.2.2.2 Periplasmic space

Unlike Gram positive bacteria, Gram negative bacteria have an area between the plasma membrane and the cell wall. This contains several hydrolytic enzymes to allow processing of incoming and outgoing molecules.

1.2.2.3 Peptidoglycan

In Gram negative organisms, peptidoglycan is only a minor fraction of the envelope but in Gram positive bacteria it may account for 30-50% of the dry weight of isolated cell walls. It may be one of the component responsible for the shape, rigidity and protective function of the walls, the latter, particularly in Gram positive organisms which do not posses an outer membrane which forms an effective barrier to a variety of substances in Gram negative bacteria.

Peptidoglycan is a heteropolymer of glycan chains containing alternative residues of

muramic acid and glucosamine in β1-4 linkage. In most bacteria these residues are N-acetylated with some exceptions. Muramic acid residues are also substituted with short peptides consisting of alternating L and D amino acids which are cross linked from one glycan to another to form a macromolecular network of high tensile strength and rigidity. In Gram negative bacteria the peptide chains have the basic structure of L Ala-D isoGlu-meso-diaminopimelic acid-D Ala and about 30-50% of these peptides are cross linked as dimers. It is the reaction of enzymes in the cross linking of glycan that gives this molecule its structure and the cell its shape. However the location of peptidoglycan as a separate layer between the inner and outer membranes of Gram negative bacteria makes it unlikely that it can react with molecules in the external environment and play a role in cell-surface interactions.

Teichoic and teichuronic acids are present in the walls of a variety of Gram positive bacteria including members of the genera *Bacillus, Lactobacillus, Streptococcus, Staphylococcus* and *Micrococcus.* They form an integral part of the wall structure and are attached to the plasma membrane (Ellwood and Tempest, 1972). Teichuronic acids are thought to replace teichoic acid when cells are grown under phosphate limiting conditions.

1.2.2.4 Outer membrane

As the outer surface of the outer membrane constitutes the outermost area of the Gram negative cell, this region is in immediate contact with the environment and has various characteristic properties. For example hydrophilic and usually negatively charged carbohydrate chains make the cell resistant to phagocytosis (Nikaido and Vaara, 1987). The negatively charged and hydrophilic nature of the surface is due to the polysaccharide chains of lipopolysaccharides and capsular polysaccharides, and also the major outer membrane proteins are all acidic proteins. The surface has considerable diversity, for example the diversity of the O antigen, which increases the chance of resistance to antibodies and digestive enzymes. The outer membrane consists of 20-25% phospholipid, 30% lipopolysaccharide and related polysaccharides and 45-50% protein.

The outer membrane has a lower phospholipid composition than the plasma membrane, however, the phospholipids are of a similar nature with a slight enrichment of

phosphatididylethanolamine (Nikaido and Vaara, 1987). Unlike the phospholipids of the plasma membrane which are symmetrically distributed, those in the outer membrane are predominantly found in the inner layer of the bilayer membrane.

Lipopolysaccharide (LPS) is a unique constituent of the Gram negative outer membrane and is not found anywhere else in the cell except as biosynthetic intermediates (Nikaido and Vaara, 1987). LPS has three distinct regions covalently linked together; the proximal hydrophobic lipid A region; the distal hydrophilic O antigen polysaccharide side chain that protrudes into the medium and the core polysaccharide region that connects the two (Figure 1.5). Each region has distinctive composition, biosynthesis and biological function. The polysaccharide side chain is the serologically dominant part of the molecule responsible for the O antigenic specificity.

The lipid A region consists of a single backbone structure of glucosaminyl β(1-4) glucosamine units substituted with 6 or 7 saturated fatty acid residues and linked to the core polysaccharide at the 3' position.

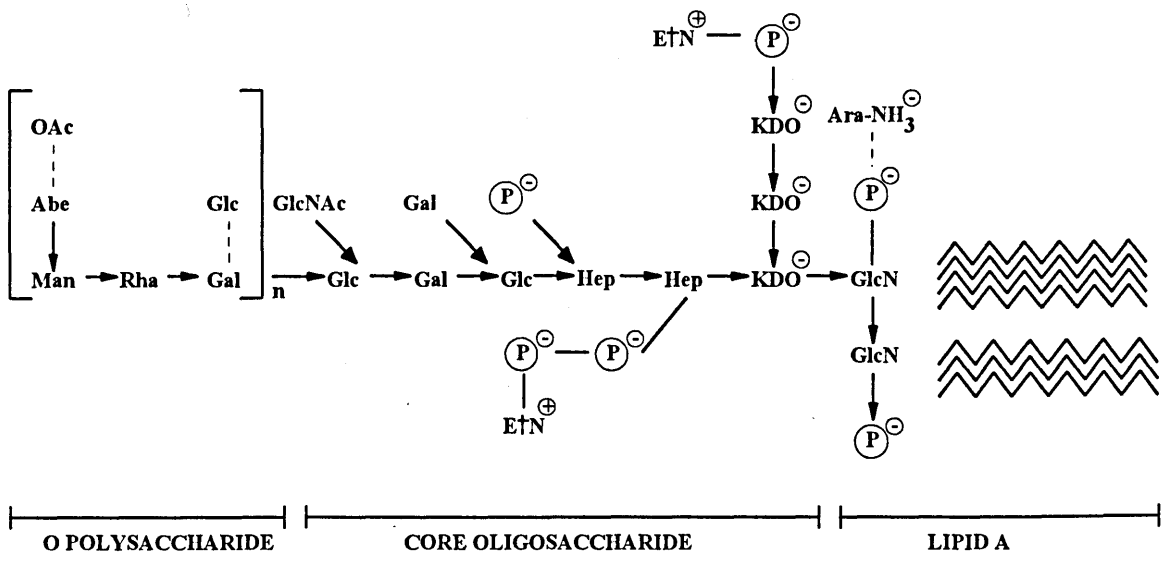
Several of the enteric bacteria including *Escherichia coli* and various *Salmonella* sp have a characteristic O antigen which is responsible for the endotoxic activity of the cell and is also the receptor of many bacteriophages. Generally the O antigen polysaccharide side chain consists of repeating oligosaccharide units, often containing rare sugars and can range in size and complexity. The enterobacterial common antigen is an acidic polysaccharide containing N acetyl-D glucosamine, N acetyl-D mannosaminuronic acid and 4-acetamido4,6-dideoxy D galactose. It represents up to 0.2% of the dry weight of an *E.coli* cell and is anchored to the outer membrane through a covalently linked phospholipid moiety.

The properties of mutants producing incomplete LPS molecules suggest the nature of the biological functions performed by these various parts of the LPS molecule. Loss of the O antigen (called rough mutants) causes a loss of virulence, suggesting that this region is important in host parasite interactions. Loss of the more proximal part of the core as in deep rough mutants results in strains that are very sensitive to a wide range of hydrophobic compounds including dyes, antibiotics, bile salts, other detergents and mutagens. Therefore this area must be important in maintaining the outer membrane as a barrier to the

environment. Mutants of lipid A assembly cannot be isolated except as conditional lethal mutants so this region must be essential to the cell; presumably it is integral in outer membrane assembly.

Figure 1.5

Structure of lipopolysaccharide (adapted from Nikaido and Vaara, 1987)



The lipopolysaccharide shown is from *Salmonella typhimurium.* Abe, abequose;Ac, acetyl; $AraNH_3$, 4-aminoarabinose; EtNP, phosphoethanolamine; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannooctulosonic acid; Rha, L rhamnose. All sugars have the D configuration unless otherwise stated. The wavy lines represent fatty acid acyl substituents on the glucosamine residues of the lipid A moiety.

The distribution of lipids in the outer membrane bilayer is highly asymmetric as the majority of the LPS molecules are located in the outer layer of the bilayer and the inner layer is composed of phospholipids. Very few phospholipid molecules are found in the outer layer. Phospholipid bilayers such as the plasma membrane are permeable to hydrophobic

molecules, but the enterobacterial outer membrane is not very permeable to such molecules. This is attributed to the asymmetric nature of the outer membrane as the LPS monolayer of the outer layer of the bilayer has low permeability. LPS contains only saturated fatty acid chains and as a consequence the hydrocarbon interior of the monolayer is less fluid than ordinary phospholipid membranes. Therefore LPS plays an important role in the barrier properties of the outer membrane which has been confirmed in studies where changes in the LPS layer resulted in increased permeability. The permeability is not altered by structural changes in the LPS, however in the case of deep rough mutants where the sugars in the proximal portion of the core are lost an increased sensitivity to hydrophobic dyes, detergents, antibiotics, fatty acids, phenols and poly cyclic hydrocarbons was observed (Nikaido and Vaara, 1987). This increased permeability could be due to a reduction in the interactions between LPS molecules or alternatively the mutation may mean that some outer membrane proteins are not inserted correctly and as a result there are increased levels of phospholipid in the outer layer so increasing the permeability. Paul *et al.* (1992) suggested that the sensitivity of *Vibrio cholerae* 569B to a wide range of chemicals particularly hydrophobic compounds and neutral and anionic detergents was due to the presence of exposed phospholipids in the outer membrane. They found a similar phospholipid profile to other Gram negative bacteria but the phospholipids were distributed differently, and the LPS moiety had a relatively low negative charge. They suggested that a combination of these two parameters was responsible for the increased permeability to exogenous compounds.

The LPS monolayer requires stabilisation by divalent cations. In the case of the smooth LPS of *E.coli* and *Salmonella typhimurium* the LPS molecules extend approximately 150 nm from the outer membrane and provide effective coverage of the other structures of the outer membrane making such structures inaccessible to antibodies and phages (Smit and Nikaido, 1978)

Due to the structure of LPS and the concentration in the outer membrane it is likely that they will greatly influence cell surface interactions.

Where the cell membrane surfaces are exposed to the environment, for example when there is reduced LPS coverage, the extrinsic membrane proteins may also be important in cell

contact and adhesion.

The outer membrane has a characteristic protein composition that is entirely different to the cytoplasmic membrane (Wicken, 1985). The outer membrane proteins (OMP) present are readily extracted using sarkosyl ionic detergent (Davies *et al.* , 1990) and the proteins separated by SDS polyacrylamide gel electrophoresis. The protein profile shows that the outer membrane consists of a few major and several minor proteins.

One of the major proteins in the outer membrane is murein lipoprotein which is a small (7.2 kDa) protein that exists in a large number of copies per cell ($7x10^5$). Approximately a third of this protein is found covalently linked to the peptidoglycan layer through the ε-amino group of the C-terminal lysine and the rest is found as free protein (Nikaido and Vaara, 1987). Mutants with deletions in the lipoprotein structural gene have unstable cell envelopes, and therefore its main function is thought to be in the stabilisation of the outer membrane peptidoglycan complex.

A further major group of proteins are the porins which form the hydrophilic channels that play a relatively non specific role in the passage of small hydrophilic molecules across the outer membrane. Many of the major outer membrane proteins are constitutively expressed but among the porins, the PhoE protein of *E.coli* is unique in that it is only produced under conditions of phosphate starvation (Tommassen and Lugtenberg, 1980). Other OMP that are induced by specific nutrient depletions include the iron regulated membrane proteins and the ButB protein receptor induced under vitamin B12 limitation. Some OMP are induced in the presence of specific nutrients, for example the Fec protein in response to the presence of citrate and the LamB protein (a maltose porin) in response to the presence of maltose.

The total amount of porins present is relatively constant and porins are the most abundant type of protein in *E.coli* and *S.typhimurium* - up to 2% of the total cell protein. Porins can also act as receptors for various phages.

The OMP of *E.coli* also include several other proteins involved in specific diffusion processes. For example there are a number of proteins involved in the transport of various chelates of ferric iron. OMP A is the most abundant porin at 10^5 copies per cell. It serves as

a receptor for various phages, spans the thickness of the membrane and can be cross linked to the underlying peptidoglycan.

Minor proteins are found in low copy number per cell and are involved in high affinity transport of specific molecules and serve as receptors for specific phages and colicins.

The OMP of a cell is very variable and dependent on the growth environment. Medium composition, pH, temperature, aeration and growth phase all affect OMP expression.

Previous work has included the characterization of the effects of chemical or enzymatic treatment of cells on adhesion. Chemicals used to denature polysaccharides include sodium periodate and disodium tetraborate and these were found to disrupt cell adhesion in attachment studies of marine (Fletcher, 1980) and freshwater bacteria. This work may indicate the involvement of polysaccharides (including lipopolysaccharides) in the attachment process as periodate oxidises and cleaves unsubstituted 1:2 glycol groups, whilst treatment with borate causes adjacent hydroxyl groups of sugars to be negatively charged altering the structure of the polysaccharides. A milder alternative has been the use of enzymes that disrupt or inhibit attachment. McEldowney and Fletcher (1986) used protease to determine the role of protein cell components in the attachment interaction and found that the enzyme did remove cells from the surface. However, the action of the enzyme is very non specific and it is difficult to draw definitive conclusions. The presence of the enzyme *per se* may also influence the attachment by altering the conditioning film.

Pringle *et al.* (1983) investigated various surface characteristics of wild type and mutant strains of *Pseudomonas fluorescens* including LPS and OMP profiles. These mutants had been isolated from a continuous culture vessel as attachment mutants. They found that there were minor changes in both the LPS and OMP profiles of the mutants compared to the wild type strain, although there were no significant differences in the major bands.

1.2.3 Exopolymers

In all groups of bacteria, strains are found whose outer surfaces are covered by a more

or less adherent layer of protein or polysaccharide. This superficial layer may be in the form of a well defined layer of hydrated polysaccharides or polypeptides, forming a capsule whose thickness may be as great as the diameter of the cell.

The phases involved in the attachment of bacteria to surfaces are described in detail in section 1.4. In summary, the bacterial cell attaches to the conditioned substratum initially reversibly and later irreversibly. Exopolymers are important in the firm irreversible adhesion of cells to surfaces and the subsequent biofilm development forming a mechanically stable highly hydrated matrix around the cells. The exopolymer matrix contains 98-99% water and consists of a variety of different polymers. It is often the biofilm matrix that causes the economic problems as it acts as a layer of immobilised water (Cooksey, 1992a). Electron microscopy of bacteria in natural habitats, particularly on solid surfaces has shown that the cells are usually associated with a fibrillar glycocalyx (Costerton *et al.* 1981).

1.2.3.1 Definitions

Costerton *et al.* (1981) proposed the use of the term glycocalyx to describe the exopolysaccharides produced by bacteria. They defined the glycocalyx as "those polysaccharide containing structures of bacterial origin, lying outside the integral elements of the outer membrane of Gram negative cells, and the peptidoglycan of Gram positive cells". Glycocalyces were further subdivided into (i) S layers which are glycoprotein subunits and (ii) capsules. Capsules were further subdivided into (a) those that are rigid and exclude particles such as India ink; (b) those that are flexible and include India ink; (c) integral capsules that are closely associated with the cell and (d) peripheral capsules that are readily lost into the aqueous medium.

However the term extracellular polymeric substances (EPS) is now more often used as it encompasses all microbes and does not only include polysaccharide containing exopolymers.

1.2.3.2 Visualisation

Electron microscopy has revealed a large number of cases in which the bacterial cell

surface is covered by a regularly packed array of protein subunits, termed an S-layer. This crystalline structure is present on a variety of eubacteria and archaebacteria, and in the case of some eubacteria is the only shape determining structure outside the plasma membrane. In the majority of Gram positive eubacteria and archaebacteria the S layers overlie the peptidoglycan, pseudomurein of sulphated polysaccharide (Lambert, 1988).

Negative staining reveals capsules that are very thick relative to the cell diameter, for example *Klebsiella* and pneumococci capsules. Thinner capsules are difficult to visualise by light microscopy. Ruthenium red and Alcian blue are polyanionic stains specific for acidic polysaccharides used in the preparation of samples for the electron microscope, but dehydration artefacts are likely due to the hydrated nature of exopolysaccharides. Stabilisation with specific anti-polysaccharide antibodies or lectins as advocated by Costerton *et al.* (1985) reduces the artefacts observed. Allison and Sutherland (1987) measured EPS production by light microscopy using congo red which is a carbohydrate specific dye.

1.2.3.3 Release of exopolymers

The mode of attachment of the capsular polysaccharide to the cell surface is unknown. Extraction of this material usually requires some physical or chemical disruption of the cell, for example phenol treatment for Gram negative bacteria and dilute acid treatment for Gram positive bacteria (Lambert, 1988). Sutherland (1982) discussed the possibility of tertiary structure formation between the capsular polysaccharide and a different cell surface polysaccharide as the mechanism for retaining the capsule at the cell surface. Costerton *et al.* (1981) on the other hand suggested that bivalent metal cations may participate as observed with some S layer assemblies.

Association of the exopolymers with the membrane is influenced by the growth conditions. Many extracellular polymers are derived from polymeric components associated with the cell surface during the earlier stages of a growth curve. The release of polymers from the bacterial surface may be determined by the micro environment surrounding the cell such as pH, the presence of endo-type capsular polysaccharide depolymerases and the structure and composition of the polysaccharide.

1.2.3.4 Composition of Exopolymers

Generally exopolymers are polysaccharides. An example of an exception is the polyglutamic capsule produced by *Bacillus anthracis* and certain other *Bacillus* strains.

As well as the sugars listed in Table 1.2, numerous unusual sugars are found in polysaccharides but generally one or more of the common sugars are present. In addition to the uronic acids, microbial exopolysaccharides often contain other types of acidic groups e.g. pyruvate. Positively charged groups are very rare in microbial polysaccharides, generally polysaccharides are hydrophilic and have a negative or zero charge. The common amino sugars are usually N-acetylated (Christensen, 1989) and a certain degree of hydrophobic character may be imparted by the acetyl and methyl groups of rhamnose and fucose.

Table 1.2

Common components of exopolysaccharides of bacteria (Christensen, 1989)

Sugars	Other components	Comments
D-glucose	O-acetyl	neutral
D-galactose	O-acetyl	neutral
D-mannose	O-acetyl	neutral
L-fucose	O-acetyl	neutral
L-rhamnose	O-acetyl	neutral
Glucosamine		Usually N-acetylated
Galactosamine		Usually N-acetylated
D-glucuronic acid	Pyruvate ketal	Negatively charged
D-galacturonic acid	Pyruvate ketal	Negatively charged

Various workers have analysed the exopolymers produced by a variety of organisms. Christensen *et al.* (1985) found that *Pseudomonas* strain NCMB 2021 produced two dissimilar polysaccharides. The first was produced in the logarithmic phase of growth and contained glucose, galactose and glucuronic acid. The second was produced in the stationary phase and contained N-acetylglucosamine, 2-keto-3-deoxyoctulosonic acid and an unidentified 6-deoxyhexose. Previous studies by Fletcher and Floodgate (1973) on the same organism had proposed the involvement of two polysaccharides in the adhesive process. They suggested that one polymer was involved in initial adhesion and was presumably on the surface of the cell, the second was thought to be produced after initial attachment and was thought to be involved in firm adhesion.

Rodrigues and Bhosle (1991) showed that a *Vibrio fischeri* strain isolated on stainless steel in sea water produced a heteropolysaccharide containing glucose and galactose as the major components. The extracellular matrix of a *Pseudomonas aeruginosa* biofilm contains protein as well as polysaccharides. In contrast, Bar-Or and Shilo (1987) found that the flocculant produced by *Phormidium* spp strain J-1 consisted of a sulphonated heteropolysaccharide of uronic acids, rhamnose, mannose and galactose to which fatty acids and proteins were bound. They also characterised the flocculant produced by *Anabaenopsis circularis* PCC 6720 as an acidic polysaccharide containing keto acid residues and neutral sugars, but no fatty acids, proteins, or sulphates were present. Reddy *et al.* (1992) analysed the capsule from *Vibrio vulnificans* and found a repeating structure with four sugar residues per repeating units which were three residues of 2-acetamido 2-6-dideoxyhexopyranose and a residue of 2-acetamido hexouronate. *Haloferax mediterrani* was also found to produce a heteropolysaccharide but contained mannose as the major component with smaller amounts of glucose, galactose, amino sugars, uronic acids and a considerable amount of sulphate (Anton *et al.* 1988). Similarly, Sutherland (1980) showed that EPS from a marine and a freshwater organism contained mannose, glucose, galactose, galacturonic acid and glucuronic acid. Plude *et al.* (1991) showed that *Microcystis flos-aquae* produced an exopolysaccharide that was similar in composition to higher plant pectin, containing galacturonic acid, rhamnose, mannose, xylose, glucose and galactose. Humphrey *et al.* (1979) grew a

Flexibacter spp on dialysis membrane and found that a polymer was produced that consisted of glucose, fucose, galactose and some uronic acids and unusually this polymer consisted of 30% protein.

1.2.3.5 Structure of exopolysaccharides

Polysaccharide structures may be divided into two types; homopolysacchrides and heteropolysaccharides. Homopolysacchrides are polymers of a single sugar or amino sugar residue e.g. glucans which are produced by the Gram positive cocci *Leuconostoc* and *Streptococci* with considerable variation in the degree of polymerisation and branching. *Streptococcus mutans* produces an insoluble glucan containing both (1-3) and (1-6) linkages as well as water soluble (1-6) glucans. These organisms have been implicated in the aetiology of dental caries, where the insoluble glucans are thought to play a role in the adhesion of these organisms to the tooth surface (Gibbons and Van Heute, 1975).

Heteropolysaccharides as the name suggests contain more than one type of sugar residue. The majority of bacterial exopolymers are heteropolysaccharides often containing uronic acids and or pyruval ketal groups, plus acetyl as common substituents. These polymers are therefore mainly negatively charged but vary in their linkages, degree of polymerisation and branching giving a variety of physical properties.

1.2.3.6 Properties

The physical properties of the polymers, especially the hydrodynamic and rheological properties are closely related to the shape of the molecules which is determined by the chemical properties (Christensen and Characklis, 1990). Allison (1992) investigated the viscosity of various polysaccharides produced by *Pseudomonas cepacia* and found that a marked increase in viscosity was observed when the polymers were mixed together. This observation may have particular relevance for mixed culture biofilms where a variety of polysaccharide types will be present. As demonstrated by Allison (1992) polysaccharides form viscous solutions and therefore the diffusional transport of dissolved molecules to and away from the biofilm surface will be at a slower rate in the presence of dissolved

polysaccharides confirming the data of Jensen and Revsbech (1989) who showed that the rate of oxygen diffusion into a biofilm was only half that in the free medium.

One important physical property that contributes to the adhesive characteristic of these polymers is their rigidity as determined from their insolubility and the high incidence of either (1-3) or (1-4) linkages in their structure. In many cases the polysaccharides are quite stiff, extended molecules (e.g. xanthan) due to their double stranded structure. Other polysaccharides are flexible due to the rotational freedom of α(1-6) linkages e.g. dextran that is produced extracellularly by several oral bacteria.

Extracellular polymers present in the aqueous phase are likely to adsorb to the surface forming a conditioning film. Pringle and Fletcher (1986) observed adsorption of bacterial exopolysaccharides and lipopolysaccharides to polystyrenes. The physicochemical effect of adsorbed polysaccharides is to change the surface characteristics and therefore bacterial adhesion.

Table 1.3

General structure of various types of microbial exopolysaccharides (Christensen, 1989)

Type	Example	
Linear homopolysaccharide	Bacterial cellulose	--Glcβ(1-4)Glcβ(1-4)Glcβ(1-4)--
	Curdlan	--Glcβ(1-3)Glcβ(1-3)Glcβ(1-3)--
Branched homopolysaccharide	Dextran	Glcα(1-6)Glcα(1-6)Glcα(1-6)-----^{1}Glcα(1-6)
Linear copolysaccharide	Bacterial alginate	--DManβ(1-4)Gulα(1-4)Gulα(1-4)--
Regular repeating units	Xanthan	(Glcβ(1-4)Glcβ(1-4))$_n$----^{1}Man----^{2}DGlu----^{3}Man

Key ; Glc, D glucose; DMan, D mannose; Gul, L guluronic acid; D glu, D glucuronic acid; Rha, Rhamnose; ^{1}side chain has α(1-3) linkage; ^{2}side chain has β(1-2) linkage; 3side chain has β(1-4) linkage.

1.2.3.7 Role of exopolymers

Zobell (1943) first suggested the involvement of EPS in the non specific adhesion of micro-organisms to surfaces and has since been supported by various electron microscopy studies using ruthenium red and alcian blue to demonstrate the presence of acidic carbohydrates (Marshall *et al.*, 1971a; Costerton *et al.*, 1981,1985; Fletcher and Floodgate, 1973). Further evidence came from the treatment of suspended or attached cells with specific carbohydrate reagents such as periodate which inhibited the attachment of a marine pseudomonad to glass (Fletcher, 1980). Fletcher and Floodgate (1973) had previously observed that a marine pseudomonad produced two exopolysaccharides. One polymer was thought to be involved in attachment to surfaces, the second was thought to be a component of the polymeric matrix between cells. Allison and Sutherland (1987) demonstrated that EPS was important in microcolony formation and therefore biofilm development but was not necessary for initial attachment. They compared the attachment of a mutant which did not produce EPS with the wild type strain and found similar levels of attachment, however unlike the wild type the mutant could not form microcolonies. Christensen *et al.* (1985) investigating the two polymers produced by a marine *Pseudomonas* spp strain NCMB 2021 concluded that the chemical structure and the solution characteristics of one of the polysaccharides correlated to some degree with the tendency of the organism to attach preferentially to hydrophobic surfaces. Generally exopolysaccharide studies are performed on the alcohol precipitate of the cell free supernatant and there is some controversy as to whether a polysaccharide that is lost from the cell can be involved in adhesion, particularly when those washed cells still attach. However the preliminary studies performed by Christensen *et al.* (1985) suggested that although exopolysaccharides were readily found in the medium, some remained associated with the cell.

Adhesion of cells to hydrophobic surfaces is commonly observed (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983). The adhesive properties of the extracellular polysaccharides were consistent with those of the corresponding bacteria, suggesting that hydrophilic exopolysaccharides in some way aided adsorption to hydrophobic surfaces. Although polysaccharides do not contain large amounts of hydrophobic groups, acetyl groups

and the 6-deoxy function in rhamnose and fucose are thought to confer hydrophobic properties.

Hermesse *et al.* (1988) reported that the presence of a visible capsule enhanced adhesion. However other workers report that extensive polysaccharide production leads to reduced attachment (Brown *et al.*, 1977, Pringle *et al.*, 1983). Similarly, Rosenberg *et al.* (1983) found that the capsule of *Acinetobacter calcoaceticus* RAG1 interfered with attachment to hydrocarbons. It is possible that different types of polysaccharide have different adhesive properties, perhaps the tightly bound exopolysaccharides are involved in adhesion, whilst more loosely associated polymers are important in microcolony and biofilm development.

It has generally been thought that it was advantageous for a cell to be attached to a surface as EPS and surfaces are thought to concentrate nutrients. However, exopolysaccharides are usually acidic polysaccharides with carboxyl and sometimes sulphate groups, which at the pH values that exist in nature could only complex with positively charged ions and very few microbial nutrients are positively charged at these pH values (Cooksey, 1992a). Characklis and Cooksey (1983) suggested that EPS production may be a response to an inhospitable environment in which the micro-organisms has become trapped and is a survival mechanism. Wrangstadh *et al.* (1990) working with *Pseudomonas* strain S9 found that it produced two exopolysaccharides. The first was closely associated with the cell and was produced during growth and starvation. The second was a loosely associated polysaccharide that was only produced in starved cells and appeared to enhance adhesion.

Various workers have reported that EPS are produced in greater amounts by surface associated organisms (Sutherland, 1980; Abu *et al.*, 1991). Beech *et al.* (1991) demonstrated that the production of EPS by *Desulfovirbrio desulfuricans* and *Pseudomonas fluorescens* was stimulated by the presence of stainless steel coupons. Similarly, Uhlinger and White (1983) found that EPS production by *Pseudomonas atlantica* was stimulated by the addition of sand particles which increased the surface area available for attachment.

Characklis and Cooksey (1983) suggested several other roles for exopolysaccharides. Exopolysaccharides may protect cells from environmental changes including the influence of

biocides, adsorb heavy metals, particulates and other detritus from the environment and enhance intercellular transfer of genetic material. Slack and Nichols (1982) suggested that the capsular material and or the extracellular mucoid substances of pathogenic bacteria protect them from antibiotics. Polysaccharides have a high charge density of fixed negatively charged groups and therefore incoming positively charged molecules including antibiotics must saturate the free binding sites in the polysaccharide matrix before penetrating to the surfaces of the cells.

Biofilms and bacterial exopolysaccharides readily bind heavy metals and therefore may be important in the cycling of trace metals and detoxification of the environment. Geesey *et al.* (1988) demonstrated that the exopolymers that anchor bacteria to metallic surfaces have a high affinity for copper ions, and this suggests that exopolysaccharides may enhance corrosion.

The environmental conditions around the cell may markedly affect the nature and amount of exopolysaccharide produced. Robinson *et al.* (1984) found that the extent of EPS production was inversely related to growth rate of *Pseudomonas aeruginosa* in continuous culture and Arad *et al.* (1988) found that the nitrogen influenced the distribution of polysaccharide between bound and soluble forms with nitrogen deficient media resulting in the highest proportion in the soluble form. Similarly, *Staphylococcus simulans* produces higher amounts of total exoprotein per unit bacterial dry weight under anaerobic conditions (Donham *et al.*, 1988). Subsequently, Bengtsson (1991) suggested that oligotrophic micro-organisms may adjust the production of EPS to the nutrient concentration of the aqueous phase possibly to promote sorption during the low nutrient conditions. Other responses to low nutrient environments that promote adsorption include the production of holdfast material by a variety of bacteria, particularly the prosthecate bacteria (Fletcher and Marshall, 1982) and the increases in cell envelope hydrophobicity observed during the minaturization of Vibrio species (Humphrey and Marshall, 1984). These adaptations reinforce the importance of surface attachment in the survival of organisms in oligotrophic systems.

1.2.4 Fimbriae

Fimbriae or pili are bacterial cell surface appendages composed primarily of a self-assembling protein subunit that is generally called pilin (also called fimbrial structural subunit). Fimbriae are helical assemblies of pilin which form a linear unbranching rod shaped structure, 2-12 nm in diameter and of variable length. They extend outwards from the cell surface and may be 0.2-10 μm in length. The number of fimbriae per cell varies from one or two to several hundred per cell. Bacterial fimbriae generally have adhesive or conjugative functions, the latter mediating the initial interaction in DNA transfer between donor and recipient cells.

Bacterial pilins are fairly hydrophobic, with hydrophobic amino acids constituting 30% or more of the pilin protein. The surface hydrophobicity of bacteria increases when fimbriae are expressed, and is particularly associated with type 1 fimbriae (Burke and Axon, 1988). It may be expected that fimbriae play an important role in adhesion as they are exposed at the surface of the cell, extend beyond the capsule and are fairly hydrophobic (Irvin, 1990).

Adhesive fimbriae are important in the adhesion of bacterial cells to various biological surfaces including erythrocytes (Old *et al.*, 1968), mammalian epithelial cells, fungi and plant roots (Duguid 1959). Bacterial fimbriae are significant virulence factors for a wide range of pathogens and antifimbrial vaccines are efficacious in preventing infections (Pecha *et al.*, 1989).

Fimbriae have been described for a wide range of Gram negative bacteria and an increasing number of Gram positive organisms. Fimbrial expression is highly dependant upon the growth condition and they are isolated by simple agitation followed by high speed centrifugation and can be analysed by polyacrylamide gel electrophoresis. Fimbriae vary in their morphology, for example *E.coli* type 1 fimbriae are fairly rigid, *Pseudomonas aeruginosa* fimbriae are more flexible and the fimbriae of *Bacteriodes gingivalis* have a corkscrew structure (Irvin, 1990).

Fimbriae are classified into several groups depending upon their characteristics. *E.coli* has Group 1 subtype 1 fimbriae that consist of a pilin subunit with a molecular weight

of 16Kda. Each cell possesses up to several hundred fimbriae peritrichously arranged. Fimbriae of Group 1 are characterised by their morphology, ability to haemagglutinate red blood cells and their sensitivity or resistance to blocking of adhesion by mannose. *Salmonella typhimurium* and several other enterobacteria posses Group 1 subtype 1 fimbriae. *Klebsiella* spp and *Serratia marcescens* have Group 1 type 3 fimbriae. However, most Gram negative bacteria have Group 2 fimbriae which have conjugative properties. Pseudomonads, *Vibrio* spp and *Agrobacterium* spp have Group 5, 4 and 3 respectively.

Isolates of *E.coli* from pyelonephritis patients can have several adhesins including type 1 P, S and F fimbriae (Arthur *et al.*, 1989). Type 1 fimbriae have been implicated in the colonisation of the catheter (Mobley *et al.*, 1988). Type 3 fimbriae of *Providencia stuartii* has been positively correlated with episodes of bacturia in catheterised patients and may promote persistent colonisation to the catheter surface (Mobley *et al.*, 1988). Both fimbrial and extracellular polysaccharide have been implicated in its adherence (McEachran and Irvin, 1985).

The examples above show that fimbriae play an important role in bacterial adhesion to viable cell surfaces, however fimbriae are also thought to be involved in attachment to inert surfaces. Irvin (1990) reported that *P.aeruginosa* fimbriae bind to both stainless steel and polystyrene in a time dependant, saturable manner with particular affinities. Irvin also concluded on the basis of monoclonal antibody inhibition studies that fimbrial-binding mediated attachment to stainless steel and polystyrene, but the domain was different to that involved in attachment to epithelial cells. The suggested mechanism involved electrostatic and hydrophobic interactions. Weerkamp *et al.* (1987) reported that the attachment of *Streptococcus salivarius* to hydrophobic surfaces depended upon the density of the fimbriae on the cell surface.

1.2.5 Prosthecae

Prosthecae are extensions of the bacterial cell that are cellular in nature and continuous with the main body of the cell itself (Morgan, 1985). Many roles for prosthecae have been proposed. In cetain forms, for example *Hyphomicrobium* and *Rhodomicrobium*

they play a role in reproduction, daughter cell formation occuring at the distal end of the prosthecae. They may also be involved in suspension by helping to maintain cells at the air-water interface. Many prosthecate bacteria attach to interfaces by means of holdfasts at the distal ends of the prosthecae. The property most commonly attributed to prosthecae is that they function as mechanisms for increasing the surface area to volume ratio of the cell, which is of particular importance in oligotrophic environments.

1.2.6 Flagella

Many bacteria can rapidly move through a liquid due to the presence of flagella that are anchored in the plasma membrane. In quiescent or very slow moving conditions motility is the main mechanism of cell movement to and from the substratum and in some cases may be involved in the attachment process (Characklis, 1990). Flagella are 10-20 nm in diameter and up to 15-20 μm long. Bacteria may move at speeds as high as 50 μm/sec. The flagella is composed of a filament, a hook and a basal body. The latter interacts with both the cell wall and plasma membrane. The most prominent extracellular portion of the flagellum is the helical filament, usually composed of the subunit flagellin which has a variable molecular weight and is species dependent.

Flagella are primarily involved in movement enabling the organism to seek available nutrients as well as avoid toxic substances by positive or negative chemotaxis. For example *Vibrio parahaemolyticus* (and other *Vibrio* sp) swarms across a surface using 100-1000 flagella (Fletcher, 1992c) and work using lux gene fusions showed that the expression of the lateral flagella gene was stimulated by the presence of a solid surface or an increase in viscosity of the medium (Silverman *et al.*, 1984, Belas *et al.*, 1986). Interference of motion of the polar flagellum, such as contact with a surface is recognised by the cell and translated into the synthesis of the lateral flagella.

Flagella may influence bacterial attachment by increasing the number of collisions with the substratum, thus increasing the probability of overcoming the repulsive forces.

Gliding motility in some Gram negative bacteria has been attributed to various mechanisms including the secretion of slime, such as the viscous glycoproteinic slime

produced by the gliding organism *Flexibacter* (Humphrey *et al.*, 1979).

1.2.7 Cell surface hydrophobicity and charge

1.2.7.1 Hydrophobicity and measurement

Measurement of bacterial hydrophobicity is important in many areas of research including biofouling, oral microbiology, phagocytosis and soil microbiology (Busscher *et al.*, 1984; Van Oss, 1978). Cell surface hydrophobicity has been implicated in a wide variety of adhesion phenomena including adhesion to muscle and fat tissue (Dickson and Koohmaraie, 1989), glass (Kjelleberg and Hermansson, 1984; Feldner *et al.*, 1983), hydroxyapetite (Knox *et al.*, 1985; Van der Mei *et al.*, 1987), stainless steel (Vanhaecke *et al.*, 1990), mineral particles (Stenstrom, 1989), plant surfaces (Smit and Stacey, 1990) and resistance of *Pseudomonas aeruginosa* to biocides (Jones *et al.*, 1989).

The term hydrophobicity is poorly understood and hydrophobic interactions between surfaces depend upon the unique properties of water. The tendency of micro-organisms to exhibit hydrophobic surface properties is determined by the complex interplay of polar and non-polar outer membrane surface components. There are numerous tests to measure cell surface hydrophobicity. Rosenberg and Doyle (1990) subdivided the range of tests into two categories. The first category of tests measure the properties of the outer cell surface as a whole and includes contact angle measurements (Busscher and Weerkamp, 1987), partitioning of cells into one or another liquid phase (Albertsson, 1958), and adsorption of individual hydrophobic molecular probes at the cell surface (Weerkamp *et al.*, 1980). The second category of tests measure hydrophobicity in terms of adhesion, for example microbial adhesion to hydrocarbons (MATH) (Rosenberg *et al.*, 1980), hydrophobic interaction chromatography (HIC) (Smyth *et al.*, 1978) and adhesion to polystyrene or other hydrophobic solid surfaces (Fletcher and Loeb, 1979). Techniques such as the salt aggregation tests (Rozgonyi *et al.*, 1985) fall between these two categories, and the direction of spread method (DOS) (Sar, 1987) does not fall in either category.

Rosenberg *et al.* (1980) using the MATH test showed that various bacterial strains

thought to possess hydrophobic surface properties adhered to liquid hydrocarbons whilst hydrophilic strains did not. The adhesion of bacteria to hydrocarbons was proposed as a simple test for studying cell surface hydrophobicity. The test is based on mixing washed cell suspensions with a hydrocarbon (e.g. n-hexadecane, p-xylene, n-octane) for a standard time and measuring the decrease in turbidity in the aqueous phase. Hexadecane is most commonly used as it has least effect on cells, and adhesion to the hydrocarbon layer is confirmed by microscope examination of droplets. This test is sensitive to the amount of surface area created when mixing the two phases and is thought to be insensitive to differences in hydrophobicity of hydrophilic bacteria (Van Loosdrecht *et al.*, 1987).

HIC measures the adsorption of cells to octyl or phenyl-Sepharose beads (Smyth *et al.*, 1978). Aqueous suspensions of the beads are packed into columns, and the percentage retention by the beads determined by the loss in turbidity or radioactivity of the eluate as compared to the original level.

The salt aggregation technique (SAT) is a relatively simple method for studying the aggregative behaviour of cells in increasing concentrations of salting out agents such as ammonium sulphate and is based on the assumption that increasingly hydrophobic bacteria will aggregate at correspondingly lower salt concentrations (Rozgonyi *et al.*, 1985). However, a limitation of this technique is that electrostatic interactions may play a role in the aggregation, and therefore the results are not a true representation of hydrophobicity alone.

Contact angle measurements are classically used to determine the surface free energy or degree of wettability of a solid surface. However the method has also been used to determine the hydrophobic properties of micro-organisms. Ideally the technique should be used on flat, smooth, dry surfaces and obviously there are certain problems applying the technique to organisms. Contact angle measurements give an overall hydrophobicity value for the cell surface, whereas a hydrophobicity value obtained by MATH may be due to a small hydrophobic portion of the cell surface mediating the adherence to the hydrocarbon phase. Surface free energies may be calculated from the contact angles and used to predict adhesion based on relative surface free energies of cells, but there are several ways to derive surface free energy from contact angle measurements. There is an inverse relationship

between surface free energy and hydrophobicity with decreasing surface energy associated with increasing hydrophobicity. Van Loosdrecht *et al.* (1987b) found that contact angles correlated well with the adhesion of bacteria to negatively charged polystyrene and concluded that contact angles were useful for estimating the hydrophobicity of the cell surface of a given organism and consequently provide an important factor for predicting its adhesion to various surfaces.

The adhesion of cells to polystyrene (or other hydrophobic surfaces) is a simple method that evaluates hydrophobicity in terms of adhesion.

The correlation between the various methods is variable. Mozes and Rouxhet (1987) compared contact angle measurements, HIC, MATH, adhesion to polystyrene and SAT. They found that *Monillella pollinis* was the most hydrophobic organism in all test methods, and that *Saccharomyces cerevisae* and *Acetobacter acetii* were the most hydrophilic organisms. However the relative hydrophobicity of the other organisms tested varied between methods. Van der Mei *et al.* (1987) compared a range of streptococcal isolates by MATH, HIC, SAT and contact angle measurements and found weak correlations between the techniques. Subsequent studies by Jones *et al.* (1991) found a good correlation between bacterial adhesion the hexadecane (BATH) and HIC, but not between BATH and SAT or between HIC and SAT.

1.2.7.2 Cell surface components involved in hydrophobicity

Surface components that may contribute to hydrophobicity include proteins and amphipathic polymers. In group A streptococci both surface M protein and lipoteichoic acid have been suggested as major contributors to both the hydrophobic properties and adherence of these bacteria to eukaryotic cells (Miorner *et al.*, 1983). Jonsson and Wadstrom (1983) found that high protein A producing strains showed a higher surface hydrophobicity than strains producing lower amount of protein A and concluded that protein A and other surface proteins such as fibronectin binding protein contribute to the high relative surface hydrophobicity. Similarly, Weerkamp *et al.* (1987) found that the attachment of *Streptococcus salivarius* to hydrophobic surfaces depended upon the density of the fibrillar

layer and surface exposure of specific types of fibril. They found that the loss of the protease sensitive fibril classes resulted in decreased ability to adsorb to hexadecane and that increased exposure of protein antigen C resulted in increased cell hydrophobicity. Evidence for the role of proteins in cell hydrophobicity include studies using proteases and protein synthesis inhibitors which result in a reduction in cell hydrophobicity (Bar-Or, 1990). In contrast, Hermansson *et al.* (1982) showed that smooth strains of *Salmonella typhimurium* and other Gram negative bacteria which carry intact lipopolysaccharide molecules are more hydrophobic than the corresponding rough mutants thus implicating LPS as mediators of cell surface hydrophobicity.

The presence of polar molecular groups on the surface of the cell favours the interaction with water molecules via hydrogen bonds and is expected to reduce the overall hydrophobicity of the surface (Mozes and Rouxhet, 1990). Mozes and Rouxhet (1990) found that hydrophobicity (as measured by HIC) was inversely related to the oxygen concentration (as measured by X-ray photoelectron spectroscopy (XPS) analysis). Mozes *et al.* (1989) found that hydrophobicity directly correlated with the fraction of the surface carbon that was attributed to hydrocarbon moieties of proteins, lipoproteins, or lipopolysaccharides and therefore hydrophobic character was not due to one specific surface component. They also reported that bacteria became more hydrophilic as the ratio of N/P increased on the cell surface, but yeasts became more hydrophobic.

1.2.7.3 Surface charge properties

The surfaces of living cells, including bacteria carry a net negative charge at physiological pH. Bacterial cells have a net negative charge at neutral pH and isoelectric points in the region pH 3. The magnitude of the charge is affected by the strain, growth conditions, pH, and the presence and concentration of various inorganic ions. The cells acquire the charge due to the ionisation of surface groups including carboxylate, amino and phosphate groups and is dependant on pH. An electrical double layer forms which extends into the aqueous region. The double layer is composed of two regions. The layer closest to the cell surface is called the Stern layer and is composed of ions of the opposite sign to that

of the surface which are retained in close contact with the surface. The potential falls linearly across this region with increasing distance from the cell surface. The second region is characterised by an exponential fall in potential, and extends much further into the aqueous phase. The surface charge properties of microbial cells are usually characterised by the zeta potential which is close to the potential at the edge of the double diffuse layer. The zeta potential is determined from the electrophoretic mobility of the cell.

As the interaction of a cell with its environment begins at the outermost layers of the cell surface, the physicochemical composition of the surface will determine whether an adjacent molecule is adsorbed or repelled (Bayer and Sloyer, 1990). The electrostatic charge of the cell surface is a net charge resulting from the combined charges of all the species that make up the cell surface and their counter-ions.

1.2.7.4 Surface charge assessment

Various techniques have been used to demonstrate net surface charge. Magnussen and Bayer (1982) labelled the anionic regions of the cell surface using positively charged molecules that were visualised using electron microscopy. Fluorescent dyes have also been used to probe the charge and potential at the bacterial surface (Aaronsen, 1981). However, electrophoretic mobilities are most commonly used to determine cell surface charge. The electrophoretic mobility of charged particles is measured in an externally applied electric field. The direction and rate of this movement depends on the polarity and density of the particles net surface charge, the strength of the electric field, the ionic strength, the temperature and pH of the medium. Electrophoretic measurements are indicative of the total charge of the cell and give no indication of the charge distribution. Micro-electrophoresis has also been used to assess surface charge and involves microscopic examination of cell electrophoresis. Isoelectric focusing has indicated that species may differ in isoelectric points as well as net negative charge (Wicken, 1985). Electrostatic interaction chromatography (EIC) is used to assess the relative surface charges of bacteria (Pederson, 1980). The technique involves assaying the degree of adsorption of bacteria to anion and cation exchange resins. The degree of affinity for the resins is dependant upon the net

surface charge of the organism.

Studies on the electrophoretic measurements of micro-organisms have provided information on the effects of physico-chemical characteristics of the environment on the cell surface and on the relationship between micro-organisms and their environment (e.g. adhesion) and the acquisition of antibiotic resistance (Collins and Stotzky, 1992).

1.2.7.5 Cell surface components involved in surface charge

In Gram positive bacteria the negative surface charge is thought to be due to the teichoic and teichuronic acids of the cell wall and the acidic polypeptides and polysaccharides of the glycocalyx (Wicken, 1985). In Gram negative bacteria the acidic lipopolysaccharides and proteins of the outer membrane and the extracellular polymers are thought to contribute to the negative charge as the outer membrane proteins have high ratios of acidic to basic amino acids and the polymers of the bacterial cell surface also contain dissociable groups such as carboxyl, phosphate, sulphate or amino groups (Bayer and Sloyer, 1990). Since most cell surfaces contain both basic and acidic groups, the surface is amphoteric with a net negative charge at high pH and a positive one at low pH. The interdependance of surface free energies, zeta potential and elemental compositions has been shown for oral streptococci (Van der Mei *et al.*, 1988), and for yeasts and bacteria (Mozes *et al.*, 1988). Mozes *et al.* (1988) concluded that the major determinants of the negative charge are the phosphate groups. However, Mamo *et al.* (1987) reported that both *Staphylococcus aureus* and various coagulase negative staphylococci had a high negative net surface charge which was due to the presence of such polymers as ribitol and glycerol teichoic acids.

1.2.7.6 Role of hydrophobicity and surface charge in cell interactions

Marshall *et al.* (1971b) suggested that both hydrophobic and electrostatic interactions are involved in adhesion of bacteria to natural surfaces. As most surfaces immersed in aqueous systems are charged it is unlikely that hydrophobic interactions are the only factor involved in determining the degree of adhesion. Van Loosdrecht *et al.* (1987b) concluded that hydrophobic cells adhered to polystyrene to a greater extent than relatively hydrophilic ones, irrespective of surface charge (as measured by electrophoretic mobility). This electrokinetic potential was apparently more important in the less hydrophobic cells. They also observed that more hydrophobic bacteria possessed a higher negative charge. The combination of high surface hydrophobicity and low surface charge would lead to such a strong adhesion that cells with these characteristics would be extremely difficult to isolate, and would be ecologically disadvantaged as spreading, dispersal and recolonization would be inhibited (Van Loosdrecht *et al.*, 1987b).

Kjelleberg and Hermansson (1984) demonstrated that certain species showed increases in both hydrophobicity and surface charge upon starvation. In contrast, Wrangstadh *et al.* (1986) found that the extracellular polysaccharide produced under starvation conditions induced a decrease in cell surface hydrophobicity and consequently the number of adhering cells However, Dawson *et al.* (1981) found that a marine *Vibrio* species became more adherent during starvation.

Allison *et al.* (1990a) found that cell surface hydrophobicity increased progressively with growth rate for planktonic, chemostat grown cells of *Pseudomonas aeruginosa* and also for cells resuspended from the biofilm. However, newly shed daughter cells were more hydrophobic and did not exhibit the same growth rate dependency. Allison *et al.* (1990a) suggested that the changes in the surface hydrophobicity were associated with the cell division cycle and that after cell division the hydrophobicity of both *P.aeruginosa* and *E.coli* cells is low. This may mean that these cells are lost from the biofilm and may play a role in the dispersal and recolonization.

Studies by Knox *et al.* (1985) confirmed that changes in the generation time, pH and carbohydrate source resulted in major changes in the organisms surface characteristics and

production of extracellular polymers and lipoteichioc acids, however, no correlation was found between adhesion to saliva coated hydroxyapetite and cell hydrophobicity. Gilbert *et al.* (1991), has however, shown that adhesiveness correlated directly with surface electronegativity and hydrophobicity for *Staphylococcus epidermidis,* but inversely with electronegativity for *E.coli.* Adhesion of hydrophilic micro-organisms such as *Saccharomyces cerevisae* and *Acetobacter acetii* are thought to be controlled essentially by electrostatic interactions (Mozes *et al.,* 1987). In contrast the adhesion of hydrophobic cells is favoured on hydrophobic supports demonstrating the importance of interfacial free energy. However, the influence of pH on the adhesion process shows that electrostatic interactions also play a role.

Dickson and Koohmaraie (1989) found a linear correlation between the relative negative charge on the bacterial cell surface and initial attachment to lean beef muscle and fat tissue, but hydrophobicity correlated with adhesion to fat tissue only. This work suggests that the interactions involved are dependent upon the nature and characteristics of the two surfaces involved.

Vanhaecke *et al.* (1990) found that the adhesion of *P.aeruginosa* to 316 L stainless steel correlated with surface hydrophobicity and concluded that cell surface charge seemed to be of minor importance in the adhesion process.

Van Loosdrecht *et al.* (1987) proposed that the adhesion of hydrophobic organisms e.g. *Staphylococci* is dominated by thermodynamic features of the cell surface such as hydrophobicity, irrespective of surface charge. For relatively hydrophilic organisms (such as *E.coli*) electrokinetic potential is the primary mediator of adhesion.

In summary, both hydrophobic and electrostatic interactions are involved in the adhesion of bacteria to surfaces. The data presented in this study examines the involvement of these two surface physico-chemical characteristics in the adhesion of various bacteria found in food processing environments.

1.3 Nature of the substratum

1.3.1 Surface charge

The majority of solid bodies acquire an electrical charge when immersed in aqueous media due to ion adsorption or ionisation of the surface groups and is dependent upon the pH of the medium (Oliveira, 1992). A charged surface immersed in an aqueous medium causes a redistribution of ions. Co-ions will be repelled from the surface whilst counter-ions will be attracted. This effect along with Brownian motion gives rise to a Poisson-Boltzmann distribution of the ions throughout the aqueous layer creating a diffuse layer which together with the solid surface is called the electrical double layer.

Electrostatic potential decreases from the charged surface through the diffuse layer, reaching zero in the bulk phase. When charged bodies approach, the interpenetration of their double layers promotes the repulsion between them. The repulsive effect is most common as the majority of solid bodies have a net negative charge in aqueous systems.

Electrostatic forces are long range forces appreciable at distances greater than 10 nm (Rouxhet and Mozes, 1990) and are thought to be important in particle deposition. As pH has been shown to affect the adhesion process, surface charge must play an important role.

Larsson and Glantz (1981) demonstrated that the adhesion of *Streptococcus sangius* to a reference metal was inhibited by treating the metal so that the surface was covered with a high density of negatively charged carboxyl groups. Treatment of the reference metal with non-polar methyl groups showed the same level of adhesion as the untreated metal. This work demonstrates the involvement of electrostatic interactions and therefore charge in adhesion.

1.3.2 Surface energy

The surface energy can be deduced from the contact angle of a liquid of known surface tension on the substratum provided that the spreading term is negligible (Rouxhet and Mozes, 1990). A measurement of the hydrophobicity is obtained if the liquid used for the contact angle measurements is water and reflects the wettability of the surface.

The free energy of adhesion between two surfaces considers the replacement of two solid-water interfaces with a solid-solid interface. The free energy of adhesion is negative, hence adhesion is favoured when the surface energies of both the solid surfaces are lower than that of the liquid medium. This occurs when both surfaces are hydrophobic and in an aqueous medium (Mozes and Rouxhet, 1992). Surface free energies depend on molecular organisation and act at short distances (<1 nm).

Busscher *et al.* (1986) showed that the adhesion of *Streptococcus sanguis* varied with surface free energy and that the organism had a low affinity for high energy surfaces. Work by Burchard *et al.* (1990) showed that the adhesion of gliding bacteria was affected by the surface energies of the substrata. Adhesion was found to be tenacious on surfaces with low critical energies (i.e. hydrophobic surfaces), whilst adhesion to hydrophilic surfaces was lower.

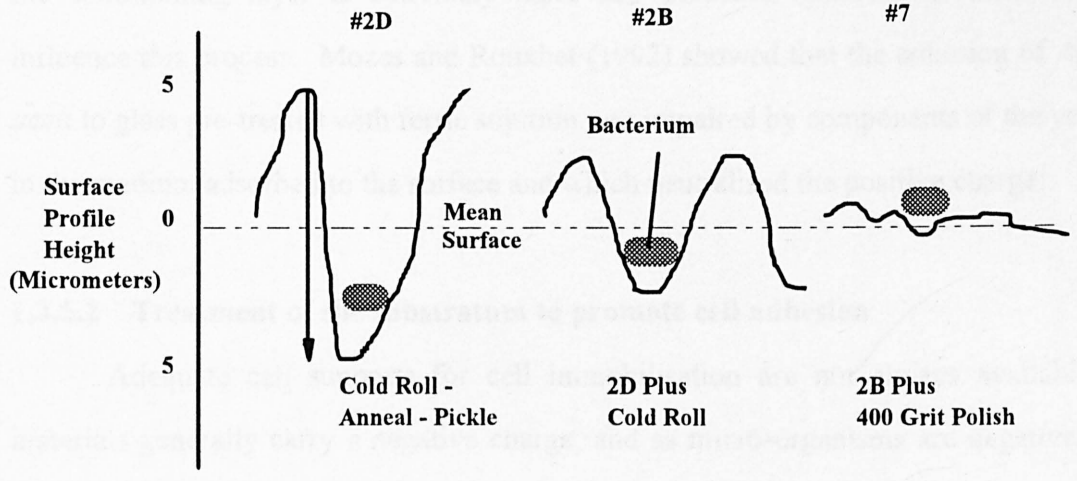
1.3.3 Surface roughness

Surface roughness is normally measured with an instrument that has a stylus that travels across the surface and the movement is amplified and recorded. The result is normally expressed in μmRa which is the arithmetic average of the deviation of the trace above its centre line (Milledge and Jowitt, 1980). Figure 1.6 shows the roughness profiles of three different mill finishes of stainless steel.

Surface micro roughness may aid cell attachment by increasing the surface area available for cell-substratum contact. In addition, cells located in the pits and crevices are sheltered from shear forces, and their removal rate is reduced (Holah and Thorpe, 1990; Mozes and Rouxhet, 1992; Stevens and Holah, 1993).

Figure 1.6

Mill finish designations for cold roll stainless steel sheet



1.3.4 Chemical composition

The chemical nature of a surface can be analysed using X-ray photoelectron spectroscopy (XPS) (Mozes and Rouxhet, 1992). The sample is irradiated with a monochromatic X ray beam which induces the ejection of photoelectrons whose kinetic energy and binding energy are characteristic of a given element.

The surface composition can be very different from that expected on the basis of the bulk phase composition. For example, the surface of polyvinyl chloride is depleted in chlorine and rich in oxygen (Mozes and Rouxhet, 1992).

1.3.5 Modification of the substratum

1.3.5.1 Adsorption of a preconditioning layer

In aqueous environments a surface is rapidly conditioned by the adsorption of organic molecules (particularly macromolecules), metallic hydroxides and hydrated oxides. In

medical and food processing environments the adsorbant molecules are generally proteins and glycoproteins (Chamberlain, 1992a). The configuration and orientation of the adsorbing molecules is influenced by the nature of the underlying substratum. Generally adsorption of the conditioning layer is extremely rapid and precedes microbial attachment and may influence this process. Mozes and Rouxhet (1992) showed that the adhesion of *Acetobacter aceti* to glass pre-treated with ferric solution was impaired by components of the yeast extract in the medium adsorbed to the surface and which neutralised the positive charge.

1.3.5.2 Treatment of the substratum to promote cell adhesion

Adequate cell supports for cell immobilisation are not always available as solid materials generally carry a negative charge, and as micro-organisms are negatively charged an electrostatic repulsion exists.

Surfaces may be modified by treatment or coating with various particles or ions. Mozes and Rouxhet (1992) describe the use of positively charged colloid particles (such as hematite and hydrous alumina), hydrolysable ions (such as Al^{3+} and Fe^{3+}), organic polycations (such as chitosan, DEAE dextran) and amine functions. These treatments all alter the surface charge in order to reduce the electrostatic repulsion and promote adhesion.

1.4 Mechanisms of bacterial attachment

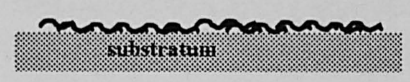
There are several phases involved in the attachment of bacteria to surfaces and the subsequent development of a biofilm. Figure 1.7 shows these phases and the following sections describe the processes involved in more detail.

1.4.1 Pre conditioning of the surface

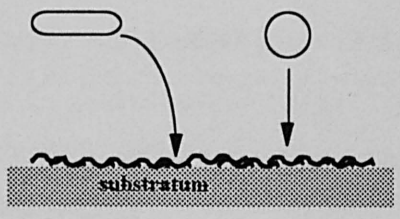
Characklis (1990) describes adsorption of an organic film as an interfacial transfer process that occurs within minutes of exposure of that surface and significantly changes the properties of the wetted surface. Little and Zsolnay (1985) measured as much as 0.8 mgm^{-2} organic matter on stainless steel after 15 minutes exposure to sea water. Similarly, Loeb and Neihof (1975) measured the adsorption rates of organic molecules to various substrata in sea water and Bryers (1980) observed analogous adsorption rates in a freshwater laboratory system. The rate at which adsorption of the conditioning layer occurs is controlled partially by the bulk concentration of the adsorbing moieties but also by their relative affinities for the surface and the hydrodynamic environment (Chamberlain, 1992a). A conditioning film forms before microbial attachment occurs due to the fact that soluble organics and small molecules are transported to the surface at a higher rate (see section 1.4.2). These conditioning films range in thickness from approximately 10 to 20 nm (Baier, 1982). The arrival of organic components at the surface is mediated by mass transfer and molecular diffusion. Unlike microbial fouling molecular fouling does not significantly effect fluid flow or heat transfer but the latter may significantly affect the microbial events that follow (Characklis and Cooksey, 1983). There is no evidence to suggest that conditioning film formation is a prerequisite for microbial attachment but because of their differential rates the surface is always conditioned before cells arrive at the surface. Fletcher (1976) reported that pretreating polystyrene with albumin, gelatine, fibrinogen and pepsin inhibited the adsorption of a marine pseudomonad possibly through steric interaction or polymer hydration phenomena (Chamberlain, 1992a).

Figure 1.7

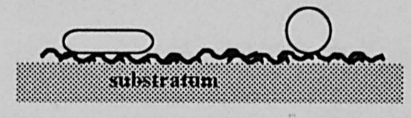
Phases in bacterial attachment and biofilm development.



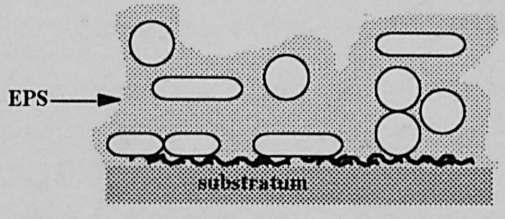
(a) Pre-conditioning - macromolecules adsorb to the substratum



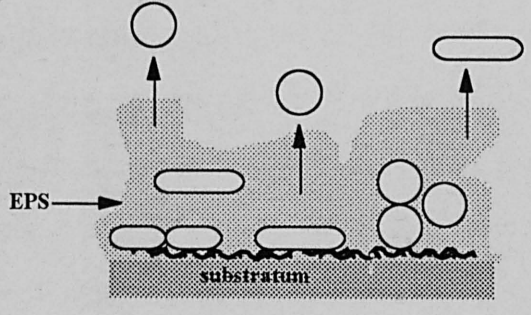
(b) Transport to the surface by diffusion , convection or active movement



(c) Adsorption to the surface



(d) Transformations - growth , reproduction and formation of metabolic products including extracellular polysaccharides (EPS)



(e) Detachment due to erosion , sloughing and abrasion

Materials with varied surface properties in terms of wettability, surface tension and electrophoretic mobility are rapidly conditioned by adsorbing organics which are often polysaccharides or glycoproteins. The nature of the conditioning components obviously depend upon the particular environment but are likely to be organic. However, metallic hydroxides, hydrated oxides and fine clay mineral particles are also found in adsorbed layers (Chamberlain, 1992b). The macromolecules bind strongly to many surfaces due to multiple attachment sites which may be either functional groups or more hydrophobic regions of the molecule. In many medical and food processing systems the adsorbing molecules are often proteins and glycoproteins, for example casein and other milk proteins bind to stainless steel and blood proteins bind to prostheses (Chamberlain, 1992a). The adsorbing species in freshwater and terrestrial systems are more heterogeneous being mainly humic components and complex polysaccharides

Little and Zsolnay (1985) demonstrated that some of the molecules adsorbed to metal surfaces in sea water desorb from the surface with exposure time despite a total net accumulation of adsorbed material.

The nature of the adsorbed conditioning layer depends upon the surface properties such as potential, charge and critical surface tension. However the substratum properties only effect the conditioning layer composition shortly after exposure. Little and Zsolnay (1985) showed that after 4 hours exposure to sea water there was little difference in the conditioning layer composition on surfaces with high surface energy such as aluminium and stainless steel. Adsorption of this conditioning layer has various effects on the substratum, including decreasing the hydrophobicity (Baier, 1975) and the zeta potentials, contact potentials and critical surface tensions are increased or decreased depending on the initial surface energy (Baier, 1975). Loeb and Neihof (1975) showed that both positively and negatively charged surfaces acquired a net negative charge. Chamberlain (1992a) reported that the adsorption of the organic components of meat juices onto stainless steel, polyethylene and polypropylene reduced the strongly negative charge of mixed oxides on alloy and imparted a net negative charge to plastics. As well as modifying the physico-chemical properties of the substratum the adsorbed layers are thought to act as a concentrated

nutrient source. Zobell and Anderson (1936) concluded that the concentration of organics on the surface resulted in enhanced microbial activity. The chemical changes that occur when molecules adsorb to the surface have been demonstrated using X-ray photoelectron spectroscopy (XPS). Mozes and Rouxhet (1992) deliberately modified surfaces by the adsorption of a variety of species including positively charged colloidal particles, ferric ions and organic polycations as measured by XPS to promote microbial adhesion. Other techniques used to analyse conditioning films include infra-red techniques and contact angle measurements which detects the change in surface free energy resulting from adsorption of the conditioning layer.

Adsorbed organic material may suppress the release of toxic metal ions, for example copper develops a surface layer of copper I oxide and small amounts of copper II ions may be released, however these are rapidly bound to adjacent organic matter (Chamberlain, 1992b).

1.4.2 Transport to the surface

Transport controls the initial rate of deposition to a clean surface immersed in natural water. In open ocean waters or in distilled water storage tanks, for example, where the concentration of both microbial cells and nutrients are low, transport of the cells to the substratum may be the rate controlling step in the biofilm accumulation for long periods of time. Hence mass transport of cells or nutrients is critical in a rate analysis of biofilm accumulation (Characklis, 1990). The mass transport processes are influenced by the extent of mixing in the bulk fluid which is dependent on the fluid flow of the system.

Bacterial cells are transported by three possible mechanisms; diffusion, convective transport and/or active movement (Van Loosdrecht *et al.*, 1990). Transport of molecules and particles smaller than 0.01-0.1 μm in either laminar flow or quiescent systems can be described in terms of diffusion. Diffusion accounts for the random contact of small bacteria with surfaces even in quiescent conditions. Diffusive transport is slow compared with transport by convective flow or the movement of motile cells (Van Loosdrecht *et al.*, 1990).

Under quiescent conditions transport of bacteria to a surface is by gravitational forces

such as sedimentation, by Brownian diffusion or by cell motility. Sedimentation rates are low for bacteria because of their size and specific gravity. Cells of 1-4 μm^3 undergo little Brownian motion, hence in quiescent systems motility may be more important (Characklis, 1990).

In laminar flow systems the mechanism for mass transport of cells in the liquid phases is diffusion and diffusion flux is proportional to the concentration gradient. The transport rate is significantly increased if the cells are motile (Characklis, 1990).

In turbulent flow systems, larger particles are transported to the substratum by fluid dynamic forces. The flux of particles to the surface increases with increasing particle concentration but is strongly dependent upon the size, shape and density of the particles. Micro-organisms are transported to the surface by diffusion, gravity, thermophoresis and fluid dynamic forces such as inertia, lift, drag, drainage and down sweeps.

Transport is also influenced by the macroscopic design of the experimental system if fluid forces vary in different micro environments within the system. Surface roughness also influences the transport rate by increasing advective mass transport near the surface (Characklis, 1990).

1.4.3 Adsorption to the surface

Adsorption of cells and other components to the substratum is an interfacial process as components leave the bulk liquid and become part of the substratum compartment. Marshall *et al.* (1971b) recognised several different mechanisms of adhesion. These include:

(i) Specific permanent adhesion; found in bacteria attaching to specific sites involving interactions between complementary molecular structures.

(ii) Non specific adhesion involving attachment of micro-organisms by for example, fimbriae and prosthecae (Corpe., 1980).

(iii) Temporary adhesion exhibited by gliding bacteria (Burchard *et al.*, 1990).

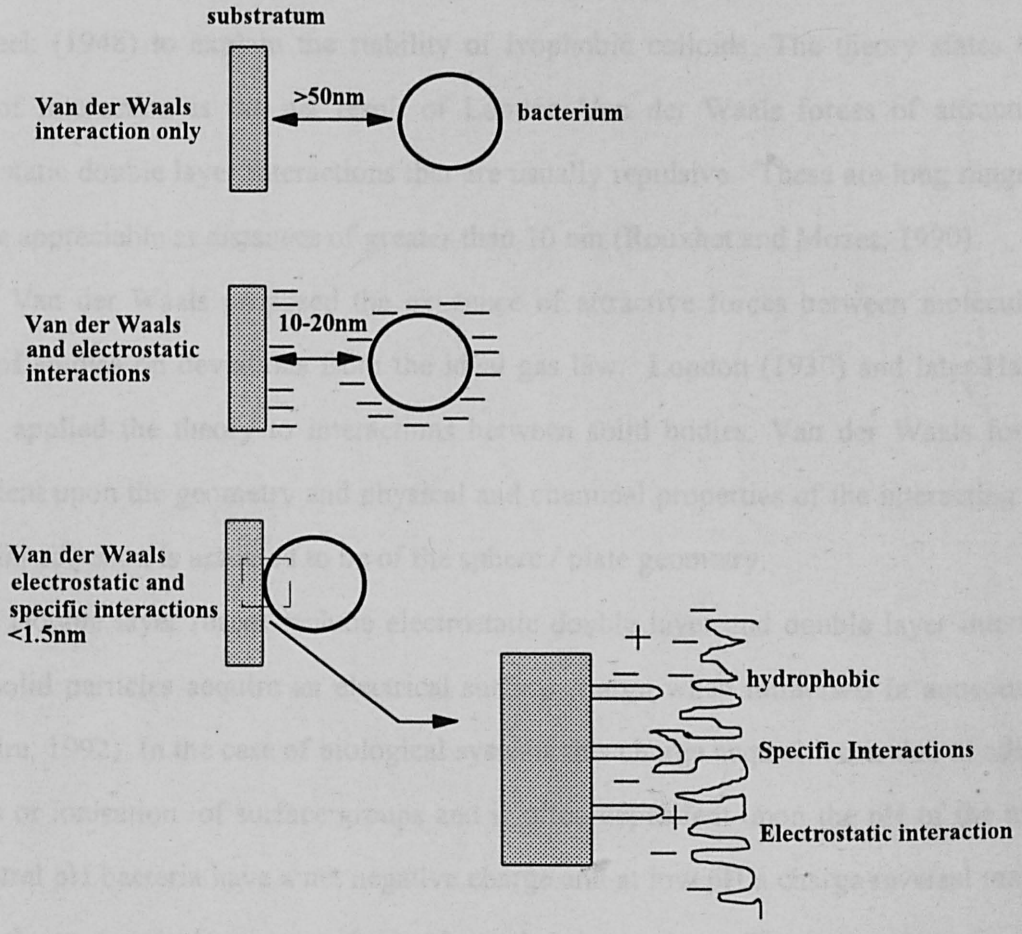
Various workers have suggested a two stage adsorption process involving reversible and irreversible adsorption (Zobell 1943; Marshall *et al.*, 1971b).

Reversible adsorption involves weak interactions between the cell and the substratum

so that the cell may still exhibit Brownian motion. Long range interactions are involved including London Van der Waals, double layer (electrostatic) and steric interactions (Characklis, 1990). At separation distances greater than 50nm Van der Waals forces operate (Figure 1.8), whilst at separation distances between 10 and 20nm, electrostatic interactions also become involved. It is thought that reversible adsorption may reflect a low specificity between cell and substratum.

Figure 1.8

The importance of various types of interaction in microbial adhesion to solid substrata (adapted from Busscher and Weerkamp, 1987).



Irreversible adsorption is a permanent bonding involving the following interactions: dipole-dipole (Keesom); dipole induced dipole (Debye); ion-dipole; hydrogen bonding; hydrophobic; polymeric bridging. Specific interactions between the cell surface and the substaratum may occur at separation distances of less than 1.5nm (Figure 1.8).

Various physicochemical models have been developed to predict microbial adhesion including the DLVO theory, surface free energy approaches and various thermodynamic models.

1.4.3.1 DLVO Theory

Microbial adsorption has been described in the literature in terms of the DLVO theory which was developed independently by Derjaguin and Landau (1941) and Verwey and Overbeek (1948) to explain the stability of lyophobic colloids. The theory states that the force of interaction is the net result of London Van der Waals forces of attraction and electrostatic double layer interactions that are usually repulsive. These are long range forces that are appreciable at distances of greater than 10 nm (Rouxhet and Mozes, 1990).

Van der Waals proposed the existence of attractive forces between molecules as a result of studies on deviations from the ideal gas law. London (1930) and later Hanmaker (1937) applied the theory to interactions between solid bodies. Van der Waals forces are dependent upon the geometry and physical and chemical properties of the interacting bodies. Bacterial adhesion is assumed to be of the sphere / plate geometry.

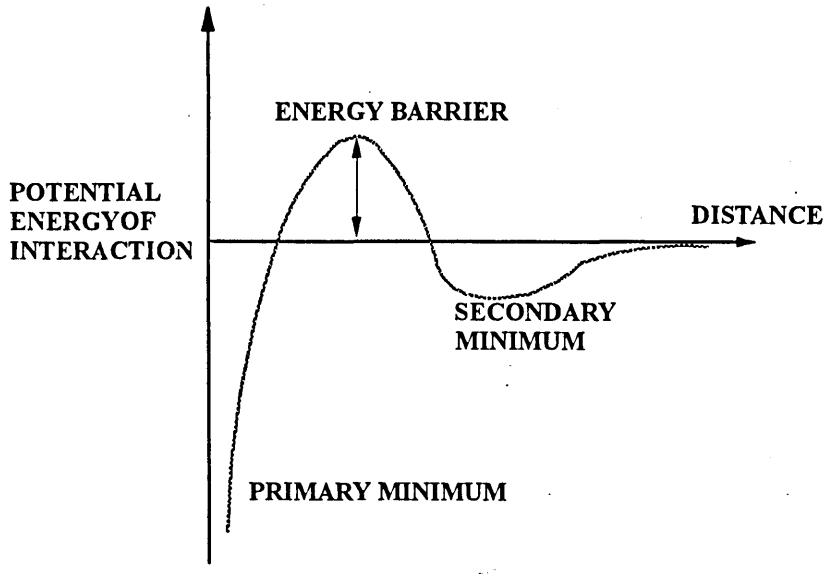
Double layer forces include electrostatic double layer and double layer interactions. Most solid particles acquire an electrical surface charge when immersed in aqueous media (Oliveira, 1992). In the case of biological systems this charge acquisition is due to adsorption of ions or ionisation of surface groups and is often dependent upon the pH of the medium. At neutral pH bacteria have a net negative charge and at low pH a charge reversal may occur due to the presence of some positively charged amino groups. The immersion of a charged surface in an aqueous medium causes a redistribution of ions so that co-ions are repelled from the surface whilst oppositely charged counter-ions are attracted. The combination of this redistribution and Brownian motion gives rise to a Poisson - Boltzmann distribution of

ions throughout the aqueous phase creating a diffuse layer which together with the solid surface is known as the electrical double layer (Oliveira, 1992). Electrostatic potential decreases from the charged surface through the diffuse layer becoming zero in the bulk phase. As the majority of solid surfaces acquire a net negative charge in aqueous solutions there is a repulsive effect between the double layers of the interacting particles. The potential energy arising from the interaction of double layers depends on the geometry of the interacting bodies and on the electrical behaviour.

The DLVO theory states that the potential energy of interaction is the sum of the energy due to Van der Waals interactions and that due to double layer interactions. Repulsive interactions are conventionally positive and attractive interactions negative. In most cases where two negatively charged particles are interacting only Van der Waals forces are attractive. Figure 1.9 gives the potential energy for this interaction.

Figure 1.9

Potential energy for interaction according to DLVO theory



The interacting bodies reach maximum stability in the primary minimum of energy. The possibility of two energy minima allows an explanation of adhesion in terms of reversible and irreversible components (Marshall *et al.* 1971b). If stabilisation occurs in the secondary minimum, cells are reversibly adsorbed to the surface and still exhibit Brownian motion and are removed from the surface by rinsing. Irreversible adsorption results if stabilisation occurs in the primary minimum, cells no longer exhibit Brownian motion and are not removed by simple washing.

The DLVO theory was developed for non living colloid particles which differ significantly from viable microbial cells that have continuous energy flux through their boundaries and have pH and proton motive gradients across the cell wall (Characklis, 1990). Considerable modifications are required in order for microbial adhesion to be described in terms of the DLVO theory. For example substratum and cell surface geometry and roughness must be considered as well as possible cell deformations that may occur. However, the DLVO theory has been relatively successful at predicting interactions at long range (>3 nm) in aqueous environments (Characklis, 1990).

1.4.3.2 Surface energy approach

Besides the long range forces accounted for in the DLVO theory, there are other forces that act at short to intermediate distances that may be attractive or repulsive and are important in adsorption, namely hydrophobic and steric interactions. Hydrophobic interactions are of a polar nature and can have a magnitude up to decimal orders higher than the components of the DLVO theory. Mozes *et al.* (1987) found that hydrophilic cells of *Saccharomyces cerevisae* did not adhere to silicate and organic polymer supports whilst the more hydrophobic organism *Monillella pollinis* adhered to the same materials. This suggested that hydrophobic interactions were involved in adhesion. Van Loosdrecht *et al.* (1987a) performed adhesion studies to negatively charged polystyrene and concluded that the electrophoretic mobility which reflects the zeta potential and surface hydrophobicity influenced the degree of coverage.

Steric forces arise between polymer coated surfaces (Oliveira, 1992) and the situation

becomes more complex when the surface layers are charged or are polyelectrolytes. In biological systems various macromolecules are present including glycoproteins, lipopolysaccharides and polysaccharides which can act as such polyelectrolytes.

Thermodynamic models have also been used to predict bacterial adhesion. Absolom *et al.* (1983) using a surface free energy approach suggested that bacterial adhesion will occur if the process of adhesion causes the free energy to decrease. Surface free energies are determined by contact angles of liquids with known surface tensions, however free energy calculations do not incorporate a contribution of electrical interactions between the surfaces which has been shown to play a role (Rouxhet and Mozes, 1990). Water is commonly used in contact angle measurements and reflects the wettability of the surface under test. The degree of wettability varies and surfaces are either hydrophobic or hydrophilic. Baier (1973) predicted that bacterial adhesion in aqueous systems would occur to surfaces with a critical surface tension of 20 to 30 mN/m. Fletcher and co-workers (1979 and 1984) found no relationship between adhesion and wettability. These conflicting results suggest that wettability alone does not predict bacterial adhesion.

1.4.3.3 Microbiological aspects

Beyond the above mentioned forces that may be involved in the adhesion process, micro-organisms reproduce, grow and produce extracellular polymers and appendages which significantly influence the adhesion process (See section 1.2). The models described above have been helpful in explaining many biological observations of microbial adhesion but these simplified models cannot take into account the adaptive changes micro-organisms readily make in response to their environment.

1.4.4 Transformations

Once a cell adsorbs to a surface or becomes attached to a biofilm, it continues its metabolic processes in response to its immediate environment. The fundamental processes include cellular growth and replication, product formation, maintenance and/or endogenous decay and cell death and lysis. Some cellular products are retained in the biofilm e.g.

extracellular polysaccharide which is important to biofilm integrity, other products diffuse out into the bulk phase (Characklis, 1990). Microbial transformations are difficult to measure directly and are normally inferred from easily observed processes such as substrate removal rate, electron acceptor (O_2) removal rate, biomass production rate, and product formation rate. Trulear and Characklis (1982) observed that the substrate removal rate increases in proportion to biofilm thickness up to a critical thickness above which the removal rate is constant, and this was attributed to diffusional resistances in the biofilm. Biofilm rate processes are controlled by mass transfer limitations in the bulk phase e.g. substrate removal rate is dependant on the fluid velocity past the biofilm. Consequently in turbulent flow systems mass transfer limitations in the liquid phase are rarely a significant factor.

1.4.5 Detachment of biofilms

Detachment occurs naturally from the moment of initial attachment and may be the loss of cells from the substratum and or biofilm matrix debris. Detachment occurs due to the following mechanisms.

1.4.5.1 Abrasion

Biofilm may be lost due to repeated collisions between substratum and particles e.g. fluidized bed reactors or in heat exchangers using cooling water with a high solids content (Bryers and Characklis, 1992).

1.4.5.2 Erosion

The continuous removal of small areas of biofilm is highly dependent on the fluid dynamic conditions (Characklis and Cooksey, 1983). Timperley (1981) and Powell and Slater (1982) studied the influence of fluid dynamics on detachment. They concluded that detachment increases with increasing Reynolds number and increasing fluid velocity. Trulear and Characklis (1982) also observed that biofilm erosion rates increased with fluid shear. Applegate and Bryers (1991) reported that the growth conditions of the biofilm strongly

of exopolymer were produced, less calcium was bound to the biofilm matrix and the erosion rate was higher than that of oxygen limited biofilms which had higher levels of exopolymer and bound calcium and consequently a more rigid morphology that was very resistant to the effects of shear.

1.4.5.3 Sloughing

Sloughing is the apparent random loss of large pieces or entire sections of biofilm and is most often observed in thicker biofilms in nutrient rich environments. Sloughing has been reported to occur due to bubble formation in either anaerobic or methane producing biofilms and nitrogen bubble formation in denitrifying biofilms (Bryers and Characklis, 1992). Sloughing can be artificially induced by the addition of ethylene glycol bis amino ethyl ether tetra acetic acid (EGTA) which chelates calcium and suggests that calcium is important in biofilm integrity. Applegate and Bryers (1991) reported that the growth conditions of the biofilm strongly affected the biofilm removal processes of erosion and sloughing. The 'fluffier' biofilm produced under carbon limitation showed higher erosion rates but never sloughed even when subjected to over 300 hours of nutrient starvation. Rigid biofilms produced under oxygen limitation showed little or no erosion but sloughing on a massive scale occurred.

1.5 Consequences of adsorption and biofilm mode of growth

1.5.1 Effect on cell activity

Zobell (1943) proposed that bacteria gain an advantage at surfaces by utilizing adsorbed macromolecules at the surface as a concentrated source of substrate. Various workers have summarised studies on the effects of surfaces on bacterial activity (Marshall, 1976; Fletcher, 1984; Van Loosdrecht *et al.*, 1990; Fletcher, 1992). Results reported by various workers ranged from stimulation, inhibition or no effect of surface attachment on the physiological activity of micro-organisms depending on the organisms, substrates and substrata involved. Mozes and Rouxhet (1992) concluded that the varied effects of surfaces on bacterial activity were due to the variability in the methods used, the types of organisms and the parameter measured as an indicator of activity e.g. growth rate, oxygen consumption, glucose consumption, carbon dioxide release, specific enzymatic activity.

The hydrodynamic conditions at the solid liquid interface are different to those of the bulk phase and dissolved solutes and particles tend to adsorb to the solid surface (Fletcher, 1992). These properties affect the concentration and flux of nutrients as well as metabolic product removal. Characklis (1984) showed that mass transfer of nutrients to the biofilm increases with flow velocity, therefore in flowing systems bacteria attached to surfaces have an ecological advantage as nutrients are delivered to the biofilm and metabolites are removed.

Adsorption of molecules or ions leads to higher concentrations at the solid liquid interface as compared to the liquid phase. Organic molecules adsorbed at surfaces are thought to serve as a concentrated source of nutrients, which may be of particular importance in low nutrient environments (Mozes and Rouxhet, 1992). Direct transfer may occur between the adsorbed layer and the fraction of the cell surface in close contact with the substratum. However, Mozes and Rouxhet (1990) concluded that because the area in contact with the surface is very small the amount of substrate present on this area of surface would be consumed within ten seconds. As well as concentrating nutrients, adsorption to the cell surface may make certain high molecular weight compounds more accessible to attached

bacteria e.g. proteins and carbohydrates which have multiple binding sites (Fletcher, 1992). Alternatively the avid binding of substrates to a surface could prevent hydrolysis by bacteria.

The solid surface may itself be a substrate and therefore attached bacteria are in direct contact with the growth substrate e.g. bacteria degrade wood, minerals and iron oxides (Holt and Jones, 1983; Munch and Ottow, 1982).

A number of mechanisms have been proposed to explain the increase in bacterial activity observed in some cases as the result of a direct effect of the surface on the bacteria. Van Loosdrecht *et al.* (1990) stated that the evidence for surfaces directly influencing bacterial metabolism was inconclusive as the observations could be attributed to the indirect effect of surfaces on the surroundings of the cells. Humphrey *et al.* (1983) and Kjelleberg *et al.* (1983) found that a hydrophilic marine bacterium showed greater size reduction and oxygen uptake when starved in the presence of a surface as compared to similarly starved cells in the absence of a surface. Later work (Humphrey and Marshall, 1984) found that the increase in endogenous respiration and the decrease in cell size was in fact due to a surfactant in the dialysis membrane used as the surface. Many bacteria produce surfactants which could accumulate at surfaces and so produce similar indirect effects of surfaces on cell size and respiration (Dagostino *et al.*, 1991). Unane *et al.* (1992) found that attached bacteria were more active than free living cells as measured by incorporation of radio labelled substrates. Iriberri *et al.* (1990) examined the specific growth rates of attached and free living bacteria in an oligotrophic marine system and found similar growth rates. Jeffrey and Paul (1986) found that the activity was greater for attached cells, as measured by thymidine incorporation and the rate of iodonitrotetrazolium reduction. Fletcher (1986) found that glucose assimilation by attached cells exceeded that of free cells by a factor of two to five times, and that the respiration of glucose by the surface attached cells was greater. Fletcher suggested two explanations for the increased activity in attached cells (a) nutrients are present in higher concentrations at the surface due to adsorption or through increased mass transfer by fluid movement over the surface and (b) modification of cell associated physiological process such as substrate transport into the cell.

1.5.2 Physiological responses to the presence of a surface

Silverman *et al.* (1984) and Belas *et al.* (1986) using lux gene fusions determined that the expression of the lateral flagella gene of *Vibrio parahaemolyticus* was stimulated by the presence not only of a surface but also by increasing viscosity of the medium. Interference with the motion of the single polar flagellum by association with a surface or an increase in the viscosity of the medium is recognised by the cell and translated into synthesis of lateral flagella. Similarly, the production of micro fibrils by *Rhizobium* and *Agrobacterium* species is a response to a signal resulting from the interaction between the bacterial cell and the plant surface (Dazzo, 1984). Karel *et al.* (1990) has suggested that contact with a surface may be sensed by membrane receptors that respond to the physical state of the cell envelope such as local loss of tension.

1.5.3 Bacterial survival in biofilms

Bacteria in biofilms are protected from certain anti-microbial agents, for example in clinical situations biofilm bacteria are less sensitive to antibiotic treatment (Anwar *et al.*, 1989). The physical barrier of the biofilm may protect the species present not only against amoebal and protozoan grazing and biocides in the environment but also macrophages, polymorphs and antibiotics in the body (Costerton *et al.*, 1987). In processing equipment and circulation systems biofilms protect microbes against cleaning and sanitation systems (Wirtanen and Mattila-Sandholm, 1992). Biofilms represent a potential problem to the food industry especially when pathogenic organisms attach to food processing equipment and remain as a source of contamination even after cleaning (Kryskinski *et al.*, 1992). Biofilm bacteria are resistant to a range of biocides including substituted phenols, biguanides, quaternary ammonium compounds and chlorine (Gilbert and Brown, 1980; Herson *et al.*, 1987). The biofilm and associated extracellular polysaccharide represents a resistance to penetration by the biocide, therefore unless the biocide can diffuse to the lower layers of the biofilm, only the microbes near the surface will be affected (Bott, 1992c). In addition crevices may not be adequately penetrated by biocides and these pockets of activity represent an inoculum for rapid biofilm growth, if the biocide treatment is not maintained on a

continuous basis. Biofilm bacteria are physiologically different from liquid phase cells and may be relatively inactive (Brown *et al.*, 1988). Disinfectants are normally tested against suspensions of test organisms, however, Holah *et al.* (1990) showed that biofilm organisms required 10-100 times the concentration of biocide to achieve log reductions comparable to those observed for suspended organisms. Blenkinsopp *et al.* (1992) found that a low strength electric field enhanced the action of several biocides against *Pseudomonas aeruginosa* biofilms. Numerous hypotheses have been proposed about the nature of this enhanced resistance in the sessile mode of growth. Early hypotheses suggested that as the EPS matrix is charged that this matrix may be responsible for binding anti-microbial agents before they reach target cells. Blenkinsopp *et al.* (1992) suggested that the observed effect was as a result of charge modification of the EPS matrix by the electric field.

Attachment to surfaces may also be a survival stategy. Kefford *et al.* (1986) found that saprophytic *Leptospira biflexa* did not undergo a major reduction in size, but starvation did result in greater adhesiveness of the organism. The authors suggest that adhesion may provide a strategy for survival of leptospires in oligotrophic habitats because the bacteria can utilise fatty acids adsorbed at surfaces. They found that the addition of an energy substrate resulted in an increase in cell size and a decrease in adhesion. Kjelleberg *et al.* (1983) and Humphrey and Marshall (1984) studied the effects of starvation on a *Vibrio* species and the activity at solid liquid interfaces. They suggested that the dwarf cells produced were a response to the interface and may be important for survival under nutrient limitations.

1.5.4 Physicochemical properties of biofilms

A biofilm is a distinct micro environment created through the development of the biofilm itself. Adsorption of macromolecules and individual bacteria is followed by the growth of bacteria, development of microcolonies, adsorption of additional bacteria and other organisms, and production of extracellular polymers. The microbial species present and the nature of the extracellular polymers determine the physical properties of the biofilm. The gel properties of the extracellular polymers dictate the transport properties of the biofilm (Christensen and Characklis, 1990). Changes in the transport rates can reduce the

effectiveness of biocide treatment (e.g. chlorination of water distribution systems). The physical properties of the extracellular polymeric gel matrix include solution properties, gel formation and adsorption to surfaces (Christensen and Characklis, 1990).

The shape of the polymer dictates its physical properties and depends upon the primary structure which is affected by pH, temperature, and the presence of inorganic salts. Aqueous solutions of EPS are usually viscous and in a biofilm the concentration of EPS is approximately 1-2% and at this concentration highly viscous.

The mechanism of gel formation has been studied for non-bacterial polymers such as agarose and alginate. Bacterial gels include curdlan, gellan gum, and bacterial alginate. These generally require the presence of cations, particularly divalent cations such as calcium and magnesium. A synergistic effect is often observed on mixing polymers resulting in the formation of stronger gels at lower concentrations than for single polymers, for example xanthan gum and galactomannans (See section 1.5.5.2).

The EPS matrix accounts for 50-90% of the biofilm organic carbon and is important for the initial interactions of cells and surfaces and biofilm integrity. The biofilm is spatially heterogeneous and the activity within the biofilm may be controlled by the diffusion of substrates or electron acceptor to the individual cells. The EPS act as diffusion barriers, molecular sieves and adsorbants. The physical, chemical and biological properties of the biofilm are dependant on the environment in which the biofilm accumulated. The dominant organism changes the micro environment in a way that is specific to that organisms metabolic activities. Although the water content of the biofilm is affected by the quantity and composition of the EPS and presence of inorganic material, the biofilm water content is in the range 87-99% (Characklis *et al.*, 1981).

The chemical compositions of EPS changes through the bacterial growth cycle. Uhlinger and White (1983) found that the relative amount of galactose in the exopolymer recovered from *Pseudomonas atlantica* decreased during the growth cycle. The anionic nature of extracellular polysaccharide gives the biofilm cation exchange properties, and therefore may concentrate cationic nutrients such as amines, especially under oligotrophic conditions (Costerton *et al.*, 1981). Particulate nutrients may also become immobilised in the

biofilm.

1.5.5 Interactions in biofilms

1.5.5.1 Succession

Successions are characterized by changes in species composition and in relative abundance of any species within a community. Allogenic succession occurs when the succession is controlled by external environmental factors, whereas autogenic succession results from the resident population altering its own environment.

Mixed species biofilm are subject to interactions such as symbiosis or competition for either space or a common substrate. The spatial distribution of microbial populations are affected by a range of selection pressures including (Bryers, 1992):

(a) The exchange of organisms with the liquid phase

(b) The efficiencies of the individual species to metabolise substrate into cell mass and extracellular polysaccharide

(c) Biofilm removal processes such as sloughing or as the result of hydrodynamic shear.

Illuminated biofilms in natural environments frequently contain bacteria and algae (Cooksey, 1992b). The interaction between the cell types is likely to be chemically mediated and in such a system it is likely that the oxygen produced by the phototrophs is used as the electron acceptor by the heterotrophs, and similarly the carbon dioxide produced by heterotrophic respiration may be utilised as a carbon source by the phototrophs.

1.5.5.2 EPS interactions

The difficulties encountered in the eradication of *Pseudomonas aeruginosa*, the causative agent of fibrocystic lung disease, has been attributed to the reduced antibiotic penetration through the EPS matrix associated with the biofilm (Allison, 1992). Allison (1992) working with *Pseudomonas cepacia*, also associated with cystic fibrotic lung, found that the composition and molecular mass of the EPS produced varied with growth conditions. The strength of interaction between two or more polysaccharides is influenced by the side

chain components. Allison observed a substantial increase in viscosity for a combined solution of bacterial and host polymers in comparison to the individual polysaccharides and a further increase was observed by the addition of calcium ions.

1.5.5.3 Genetic transfer

The close proximity of cells in the biofilm may promote genetic exchange (Characklis and Cooksey, 1983). However, Simonsen (1990) suggested that as bacteria in biofilms grow as microcolonies that are fixed in space that the cells do not have equal opportunities in mating events, and concluded that dynamics of plasmid transfer on surfaces was quantitatively different to that of liquid phase cells. In contrast, Lorenz *et al.* (1988) found the transformation frequency to be 25-50 times higher in *Bacillus subtilis* cells attached to sand grains as compared with liquid phase cells.

1.6 Summary of the methods used to analyse attachment and biofilm development

The methods and techniques that have been used to model attachment and biofilm development, quantify the attached population, analyse exopolysaccharides, cell surface hydrophobicity and charge, and the nature of the substratum are summarized in the following tables (Tables 1.4-1.9).

Table 1.4

Systems used to model attachment and biofilm development.

Method	Reference
Static adhesion assays	Fletcher (1976)
Chemostat systems	Molin and Nilsson (1983)
Rotating annular reactors	Trulear and Characklis (1979)
Flow cell reactors - tubular flow cells	Bryers (1980)
Flow cell reactors - parallel plate flow cells	Bryers and Rudoll (1992)
Radial flow reactor	Fowler and McKay (1980)
Perfused filter system	Gilbert *et al.* (1991)
Constant depth laboratory film fermenter	Peters and Wimpenny (1988)

Table 1.5

Summary of the methods used to analyse the attached polulation

Method	Reference
Light microscopy	Allison and Sutherland (1987)
Epifluorescent microscopy	Holah *et al.* (1988)
Computer enhanced microscopy	Lawrence and Caldwell (1987)
Immunofluorescent microscopy	Zambon *et al.* (1984); Spenceley *et al.* (1992)
Interference reflection microscopy	Fletcher (1988)
Scanning electron microscopy	Lewis *et al.* (1987)
Transmission electron microscopy	Marshall *et al.* (1971)
Scanning confocal laser microscopy	Lawrence and Caldwell (1987)
Detection of structural components - ATP	Harber *et al.* (1983)
Detection of structural components - DNA	Paul and Loeb (1983)
Detection of structural components - LPS	Dexter *et al.* (1975)
Radiotracer labelling	Pringle and Fletcher (1983)
Nucleic acid probes e.g. 16S RNA probes	Holbern *et al.* (1988); Amann *et al.* (1992)
Thickness measurements	Characklis (1990)
Activity analysis - radiolabelling	Paul and Jeffery (1986)
Activity analysis - lux genes	Walker *et al.* (1992)
Impedence techniques	Holah *et al.* (1990)
Microelectrodes	Van den Heuvel *et al.* (1992)
Heat and flow resistance measurements	Bott (1992)
Infra red monitoring systems	Pujo and Bott (1992)

Table 1.6

Methods used to analyse exopolysaccharides

Method	Reference
Alcohol precipitation	Beech *et al.* (1991)
Dialysis isolation	Ha *et al.* (1991)
Total sugar assay	Dubois *et al.* (1956)
Uronic acic assay	Blumenkrantz and Absoe Hansen (1973)
Thin layer chromatography	Bar-Or and Shilo (1987)
Nuclear magnetic resonance spectroscopic analysis	Reddy *et al.* (1992)
Interference reflection microscopy	Marshall *et al.* (1989)
HPLC/GC	Uhlinger and White (1983); Rodrigues and Bhosle (1991); Kennedy and Sutherland (1987)
Ruthenium red staining	Fletcher and Floodgate (1973)
Congo red staining	Allison and Sutherland (1987)

Table 1.7

Methods used to assess cell surface hydrophobicity

Methods	Reference
Microbial adhesion to hydrocarbons (MATH)	Rosenberg *et al.* (1980)
Hydrophobic interaction chromatography (HIC)	Smyth *et al.* (1978)
Salt aggregation technique (SAT)	Rozganyi *et al.* (1985)
Direction of spreading (DOS)	Sar (1987)
Two phase partitioning	Albertsson (1958)
Contact angle measurements	Busscher and Weerkamp (1987)

Table 1.8

Methods used to assess cell surface charge

Method	Reference
Electrophoretic mobility	James (1991)
Isoelectric focussing	Wicksen (1985)
Electrostatic interaction chromatography	Pederson (1980)
Fluorescent labelling	Aaronsen (1981)
Immunolabelling	Magnussen and Bayer (1982)

Table 1.9

Methods used to analyse the substratum

Method	Reference
Surface roughness	Milledge and Jowitt (1980)
Contact angle measurements to estimate surface free energy	Busscher and Weerkamp (1987)
Xray photoelectron spectroscopy charge	Mozes and Rouxhet (1992)

1.7 Aims

The overall objective was to examine attachment and biofilm development of organisms found in food production environments to stainless steel. A basic understanding of the attachment of organisms in the food environment is the first step in being able to reduce or eliminate the problem or alternatively develop approaches to achieve this end.

The aim of initial work was to isolate and characterize the organisms found on surfaces in the food processing environment in order to select a mixture of organisms to model mixed culture biofilm development in the laboratory. This work lead into the examination of cell surface characteristics to explain the differences in the attachment abilities of the three species involved in the biofilm consortium including outer membrane proteins, exopolysaccharides, lipopolysaccharides, cell hydrophobicity, charge, antigenicity and cell volume changes.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Bacterial isolates

Environmental isolates were required to model biofilm development using organisms that occured naturally as a consortium in a food processing environment. A range of product-type environments were examined (Table 2.1).

Table 2.1

Factory environments sampled

Factory environment	Products present
Supermarket preparation area	Raw and cooked fish and meats
Supermarket cafeteria kitchens	Raw and cooked foods
Abattoir	Fresh and frozen pork
Salmon factory	Smoked salmon

Stainless steel surfaces from production and preparation areas were swabbed using cotton swabs (Sterilin). The swabs were transported in 9 ml phosphate buffered saline (PBS) plus 1ml inactivator at 4°C. The inactivator was included to neutralize any residues that could have affected cell viability and contained the following (per litre) :-

Soya lecithin	3.0g
Tween 80	30ml
Sodium thiosulphate	5g
L-histidine	1g
0.25M Potassium dihydrogen phosphate (KH_2PO_4)	10ml

The inactivator was sterilized by autoclaving at 121°C for 15 min. In the laboratory organisms were recovered from the swabs by vortexing in phosphate buffered saline (PBS) which contained the following (per litre) :-

Sodium chloride (NaCl)	8.0 g
Potassium chloride (KCl)	0.2 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g

The pH was adjusted to 7.4 and the media sterilized by autoclaving at 121 °C for 15 min.

Serial dilutions were made in PBS and 100 µl of each dilution was spread onto Violet Red Bile Agar (Oxoid) for coliforms, Baird Parker (Oxoid) for staphylococci, nutrient agar (Oxoid) for total viable count and Blood agar (Difco) for fastidious organisms.

2.2 Bacterial identification

Bacteria were examined by light microscopy using a Leitz microscope (section 2.11) fitted with phase contrast optics to determine cell morphology and motility. The organisms were further characterised by Gram reaction and oxidase and catalase tests. Finally identifications were taken to the species level using API Identification kits (Bio Mérieux, France). The API systems are standardized, minaturized versions of the conventional procedures for bacterial identification. API 20E was used for Enterobacteriaceae, API Staph for *Staphylococci*, API Strep for *Streptococci* and API 20NE for non Enterobacteriacea. A single colony of each strain to be tested was resuspended in 5ml of sterile distilled water and used to inoculate the test strips, which were incubated at 37°C for 18-24 hr. The addition of reagents and interpretation of the results were as described by the manufacturers. The pattern of reactions was then coded into a numerical profile and identification made with the computer based API Profile Recognition System.

2.3 Choice of organisms

The biofilms isolated ranged from single species biofilms to complex multi-species mixtures of up to twelve organisms. A relatively simple mixture of three species found together on the same stainless steel surface in the salmon factory was chosen to model biofilm development in the laboratory. The bacterial species used in all studies were :-

Serratia liquefaciens

Staphylococcus cohnii

Pseudomonas fragi

2.4 Media

2.4.1 Luria Bertani (LB) Broth

Cultures were grown routinely in LB which contained the following (per litre);

Yeast extract (Oxoid)	5 g
Sodium chloride (NaCl)	10 g
Bactotryptone (Difco)	10 g

The pH was adjusted to 7.5 with sodium hydroxide (NaOH) solution and the media sterilised by autoclaving at 121°C for 15 min.

2.4.2 Minimal salts media

2.4.2.1 Carbon (C) limited

The following were added (per litre);

Disodium hydrogen phosphate (Na_2HPO_4)	2.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Ammonium chloride (NH_4Cl)	1.9 g
Ammonium nitrate (NH_4NO_3)	1.9 g
Disodium sulphate (Na_2SO_4)	2.0 g
Magnesium sulphate ($MgSO_4$)	0.1 g

The pH was adjusted to 7.4 and volumes less than 2 l were autoclaved as the above mixture. However, for larger volumes it was found that the longer autoclave cycle resulted in the precipitation of the phosphates consequently these were added after autoclaving as filter sterilised solutions.

Filter sterilised glucose was added to give a final concentration of 0.1% (w/v).

2.4.2.2 Nitrogen (N) limited

The following were added (per litre);

Disodium hydrogen phosphate (Na_2HPO_4)	2.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Ammonium chloride (NH_4Cl)	0.1 g
Ammonium nitrate (NH_4NO_3)	0.1 g
Disodium sulphate (Na_2SO_4)	2.0 g
Magnesium sulphate ($MgSO_4$)	0.1 g

The pH was adjusted to 7.4 and volumes less than 2 l were autoclaved as the above mixture. However, for larger volumes it was found that the longer autoclave cycle resulted in the precipitation of the phosphates so these were added after autoclaving as filter sterilised solutions.

Filter sterilised glucose was added to give a final concentration of 0.5% (w/v).

2.4.2.3 C and N excess

The C limited medium (section 2.4.2.1) which contained excess levels of nitrogen was used but filter sterilised glucose was added at the higher concentration of 0.5% (w/v).

2.4.2.4 Dialysis of yeast extract

In certain exopolysaccharide experiments dialysate of yeast extract was added at 0.1% (v/v). It was necessary to dialyse the yeast extract so that the polymers that would interfere with the polysaccharide assay were retained in the dialysis bag. The dialysate was obtained by placing 10% (w/v) sterile yeast extract in a dialysis bag (molecular weight cut 1000kDa)

and dialysing overnight in an equivalent volume of sterile distilled water. The dialysate was then filter sterilized.

2.4.3 Agar

Nutrient agar (Oxoid) was used for routine growth and maintenance.

2.5 Batch culture

2.5.1 Aerobic

Cultures were grown aerobically at 30°C in a Gallenkamp Orbital Shaker (200 rpm) unless stipulated otherwise. Liquid cultures were propagated overnight by inoculating 10 ml medium in 25 ml universal bottles, with a single bacterial colony from an agar plate or in certain experiments with 100 µl of a fresh overnight culture. A variety of glass culture vessels were used with capacities ranging from 25 ml to 2 litres.

2.5.2 Anaerobic

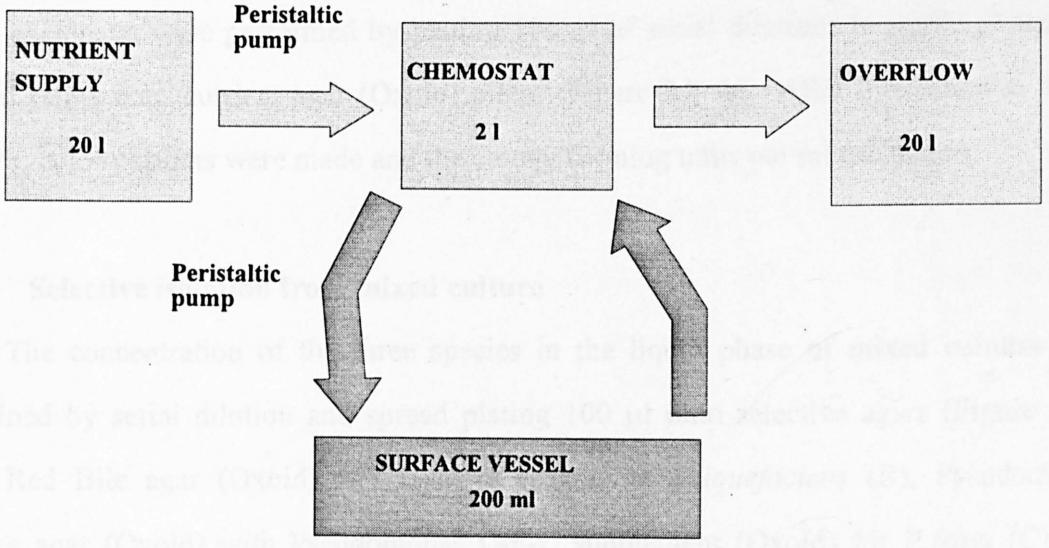
Anaerobic conditions were obtained by sealing the flasks with subaseals and flushing with oxygen-free nitrogen for 15 min via sterile syringe needles inserted through the subaseal.

2.6 Chemostat culture

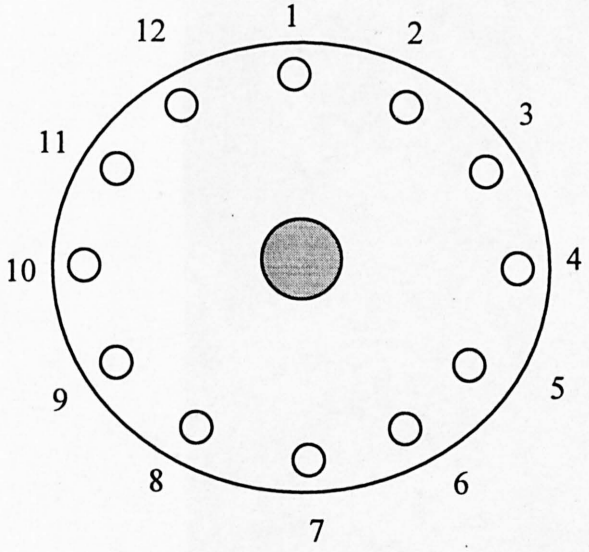
The bacterial species were grown as pure cultures or as a three species mixed cultures in a 2 l chemostat (LH fermenter) in either C or N limited media at dilution rates between 0.02 and 0.2 hr^{-1}. The temperature was maintained at 25°C .Cultures were aerated at a rate of 1.5 l min^{-1} and stirred at 500-750 rpm. The pH was maintained at 7.4 by the automatic addition of 1M potassium hydroxide (KOH) or 1M sulphuric acid (H_2SO_4). Surfaces were introduced to the system as described in section 2.12.

Figure 2.1

A. Flow Diagram of the chemostat system



B. Schematic diagram of chemostat top plate



1 Nutrient Supply
2 pH Probe
3 Oxygen Probe
4 Temp Probe
5 Overflow to waste
6 pH Control - Acid or Alkali Addition
7 Recirculation loop to surface vessel
8 Sample Port
9 Heating Element
10 Air In
11 Vent to air filter
12 Re-circulation loop from surface vessel

2.7 Viable count determinations

2.7.1 Total viable count

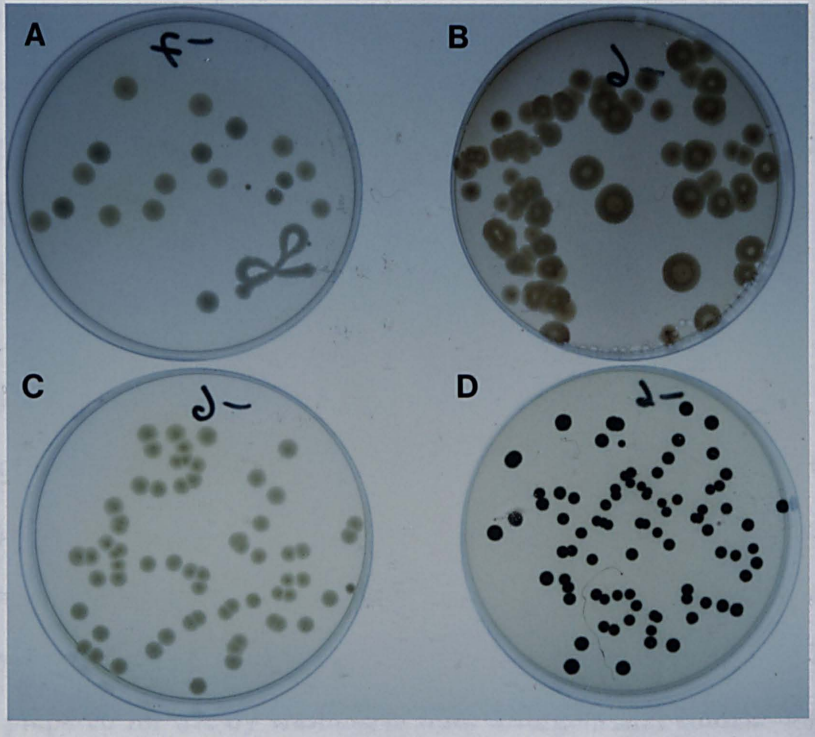
Routine determinations of culture viability and total viable counts from the mixed species chemostat were performed by plating 100 µl of serial dilutions in sterile phosphate buffered saline onto nutrient agar (Oxoid) plates (Figure 2.2 A). After incubation at 30°C for 48 hr, colony counts were made and the colony forming units per ml calculated.

2.7.2 Selective isolation from mixed culture

The concentration of the three species in the liquid phase of mixed cultures was determined by serial dilution and spread plating 100 µl onto selective agars (Figure 2.2). Violet Red Bile agar (Oxoid) was used to enumerate *S.liquefaciens* (B), Pseudomonas selective agar (Oxoid) with Pseudomonas C-F-C supplement (Oxoid) for *P.fragi* (C) and Baird Parker (Oxoid) for *S.cohnii* (D). Plates were incubated at 30°C for 48 hr.

Figure 2.2

Colony morphology on the selective media



2.8 Maintenance of cultures

To avoid laboratory attenuation of the isolates, exponential phase cultures were stored at -20°C in 15% (v/v) glycerol. Organisms were streaked onto nutrient agar from the frozen stocks once a month to avoid prolonged subculture.

2.9 Purity of cultures

The purity of the cultures was always checked prior to use by phase contrast microscopy. Purity of liquid phase and stock cultures was also checked by streaking onto nutrient agar plates which were incubated at 30°C overnight.

2.10 Spectrophotometric determinations

Spectrophotometric determinations were performed using a Shandon double beam UV 150-02 spectrophotometer. Plastic cuvettes with a 1 cm light path were routinely used. Culture turbidity was determined at a wavelength of 600 nm.

2.11 Light microscopy

Light microscopy was carried out using a Leitz Dialux 22/22 EB microscope with phase contrast optics and fitted with a Leitz Vario Orthomat 2 automatic camera. Photomicrographs were taken on Kodak T-max 100 film which was developed in Kodak Detkol developer for 5 min at 20°C and fixed in Kodak Unifix. Prints on Kodak Veribrom paper were developed with Ilford contrast FF developer and Kodak Unifix.

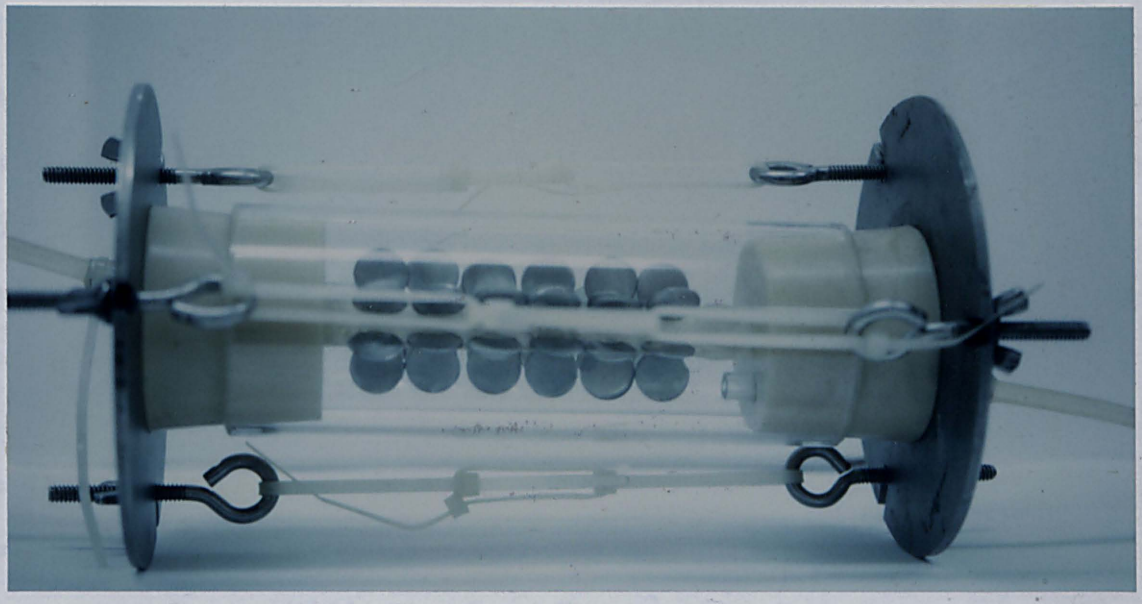
2.12 Biofilm development

Monospecies and mixed species biofilm development was modelled by introducing surface samples to the chemostat system on a loop from the chemostat vessel (Figure 2.1). Stainless steel discs were routinely used, although glass coverslips (13 mm diameter) were used initially. The discs were type 316 stainless steel with a 2B finish, 13 mm in diameter which were prepared for use by washing in mild detergent and autoclaving at 121°C for 15 min. The vessel containing the surfaces comprised a glass cylinder (15 cm length, 5 cm

diameter) with a silicone bung in either end (Figure 2.3). Down the centre of the cylinder was a glass rod inside a piece of silicone tubing which was held in place by pushing the ends of the rod into the silicone stoppers. Slits were made in the silicone tubing around the rod to hold the glass coverslips or stainless steel discs so that the liquid flow was across the surfaces. The whole assembly was clamped together. The surface contact vessel was introduced to the chemostat system once steady state had been reached. The culture was subsequently pumped through the surface contact vessel at a rate of 100 ml min^{-1}. Surfaces were removed at intervals and the numbers of attached cells enumerated as described in sections 2.14 and 2.15.

Figure 2.3

Vessel containing stainless steel discs



2.13 Static Adhesion Assay

Cells were harvested by centrifugation at 4800 g for 15 min, washed twice in PBS and resuspended in PBS to give an absorbance reading of approximately 0.3 at 600 nm (3.8×10^8 cells ml^{-1} for *S.liquefaciens*, 5.41×10^8 for *P.fragi* and 2.78×10^8 for *S.cohnii*). Accurate cell concentrations were determined by serial dilution in PBS and spread plating 100µl onto nutrient agar (section 2.7.1). Triplicate sterile stainless steel discs were immersed in the washed cell suspensions for 1 hr at 25°C. Preliminary studies showed that an hour was a suitable attachment period, as attachment did not increase significantly with longer incubation periods. Attached bacteria were enumerated as described in section 2.14.

2.14 Direct epifluorescent microscopy using Acridine Orange staining

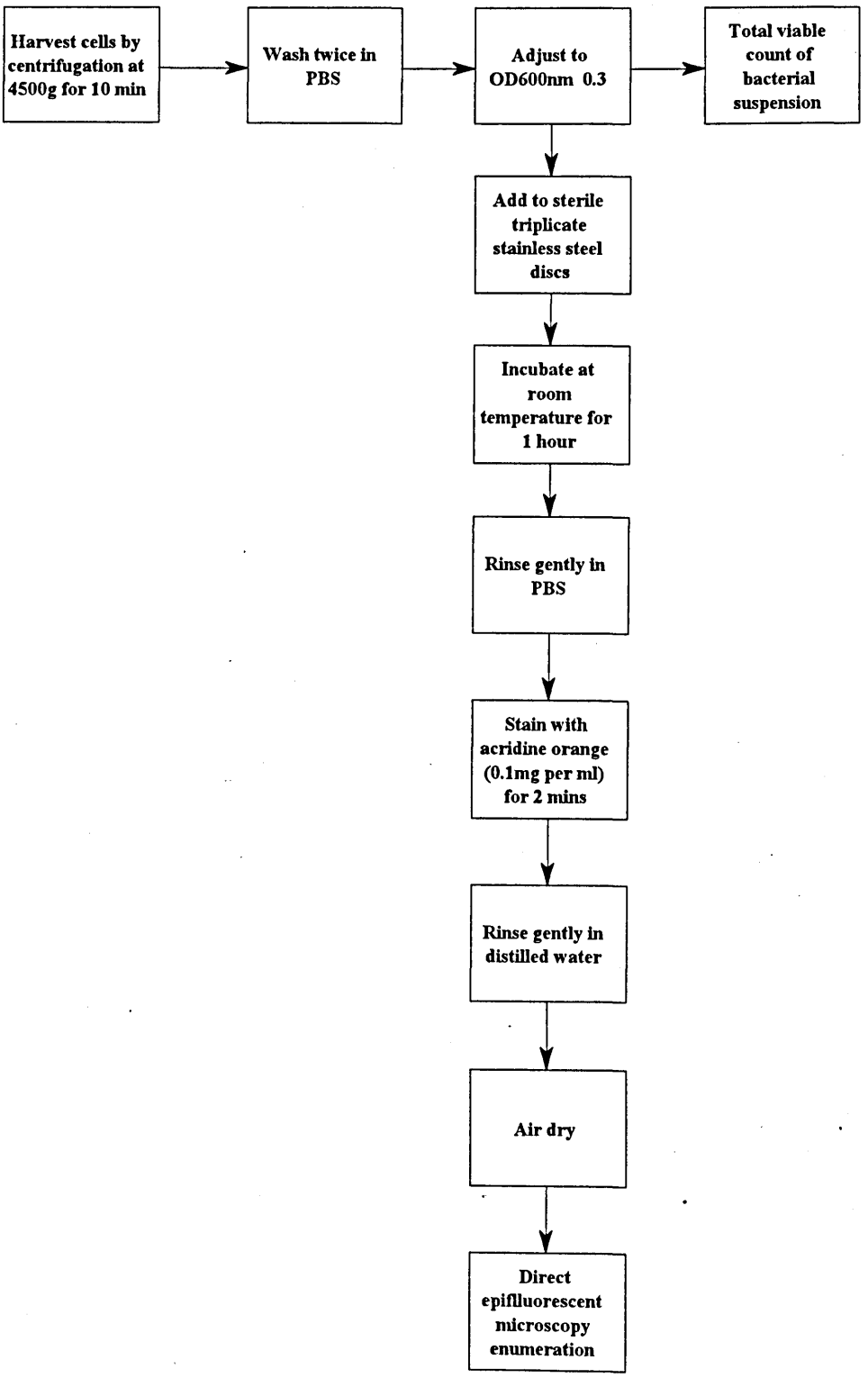
Acridine orange staining was used to determine the numbers of attached bacteria from single species experiments or to determine a total attached bacteria count from mixed culture biofilm development. Surface samples were washed twice in PBS and stained in 0.1 mg ml^{-1} acridine orange in PBS for 5 min. The surfaces were washed in PBS and air dried. Attached bacteria were enumerated by direct epifluorescent microscopy linked to an Optimax image analysis system (Holah *et al.* ,1988). The system has an automatic stage so that 20 fields of view are analysed for the area covered by fluorescent cells. As the system was calibrated for the mean cell area of one bacterium the system can calculate the concentration of cells on the surface in cell cm^{-2}.

Attachment ratios were calculated to allow for differences in liquid phase cell concentrations by dividing the surface count in cells cm^{-2} by the suspension concentration in cells ml^{-1}.

$$\text{Attachment ratio} = \frac{\text{cells cm}^{-2}}{\text{cells ml}^{-1}}$$

Figure 2.4

Flow diagram of static adhesion assay



2.15 Immunofluorescent labelling of attached bacteria

Stainless steel discs removed from the fermenter system were washed twice in PBS and fixed in 1% (v/v) glutaraldehyde in PBS for 1 hr at room temperature. The surfaces were washed twice in PBS and then permeabilized with 0.2% (v/v) Triton X-100 in PBS for 2 min at room temperature (Harlow and Lane, 1988). The surfaces were rinsed gently in PBS with 4 changes over 5 min, and placed on parafilm covered, damp filter paper in a humid chamber. The primary antibody to each organism (section 2.28) was diluted 1:100 for *S.liquefaciens* and *P.fragi* and 1:50 for *S.cohnii* in PBS containing 10% dry milk (Marvel) . The first primary antibody was added to the surfaces for a minimum of 30 min at room temperature. The surfaces were then washed with 3 changes of PBS containing 1% (v/v) Triton X-100 over 5 min. The secondary antibodies used were anti-rabbit immunoconjugates of fluorescein isothiocyanate (FITC) (Sigma), tetramethylrhodamine isothiocyanate (TRITC) (Sigma) and Texas Red (Americium). The secondary antibodies were diluted 1:200 in PBS containing 10% dry milk and added to the surfaces for a minimum of 20 min at room temperature. The surfaces were washed in 3 changes of PBS over 5 min. If required, a second primary antibody was added at this stage for a minimum of 30 min, the surfaces washed and a secondary antibody which emits at a different wavelength was added. Finally the third primary antibody was added and linked to one of the immunoconjugates. Staining the two rod shaped organisms with FITC and TRITC enabled them to be differentiated on the basis of the fluorescent tagging, whilst the coccoid organism could be differentiated on cell morphology. Control surfaces were used to check that the secondary antibodies did not bind non specifically to the organisms.

Attached species were enumerated using a fluorescent microscope (Olympus) and an eye piece graticule. The concentration of cells on the surface was calculated as cells cm^{-2}. Attachment ratios were calculated to allow for differences in the liquid phase cell concentration as described in section 2.14.

2.16 Scanning electron microscopy

Surface samples removed from the fermenter system were gently washed in PBS and the attached population fixed in 4% (v/v) glutaraldehyde in PBS for 2 hr at room temperature, as described by Lewis and Gilmour (1987). The surfaces were washed in PBS by immersion for 10 min and dehydrated by passage through a graded alcohol series from 10%;25%;50%;70%;90; to 100% (v/v) ethanol for 10 min in each concentration. The samples were then critical point dried. Gradually ethanol was replaced with amyl acetate by changing from neat ethanol to 1:3 amyl acetate:ethanol, to 1:1 amyl acetate:ethanol, to 3:1 amyl acetate:ethanol and finally to neat amyl acetate. The residence time in each concentration was 10-15 min. The sample was subsequently transferred under amyl acetate to the cooled critical point drier. Amyl acetate was replaced with liquid carbon dioxide, after which the temperature was increased to above the critical point so that the carbon dioxide evaporated. After critical point drying the surface samples were mounted on stubs (Agar; 2.5 cm aluminium pin stubs) and sputter coated with gold for 120 sec using a Biorad Polaron Division E5200 Auto sputter coater. The surfaces were examined using a Joel JSM T330A scanning electron microscope. Photographs were taken using a Polaroid camera using Polaroid type 55 positive negative film.

2.17 Transmission electron microscopy

2.17.1 Negative staining

Samples were examined after negative staining with phosphotungstic acid in the following manner. A drop of cell suspension was placed on a Formvar coated grid (Agar 100 segment mesh ; 3.05 mm diameter) for 30 - 60 sec, then the excess liquid was removed with a strip of filter paper. The grid was allowed to dry and the sample negatively stained by placing a drop of 1% (w/v) phosphotungstic acid (pH 7) onto the grid and removing it immediately with a strip of filter paper.

Specimens were examined using a Joel JEM-100S transmission electron microscope operating at an accelerating voltage of 80 Kv. Photographs were taken using Kodak 4489

Estar thick base electron microscope film which was developed in Kodak D19 developer and fixed in Kodafix according to the manufacturer's instructions.

2.17.2 Heavy metal shadowing

Samples were introduced to the grids as described in section 2.17.1. The samples were air dried and attached by their edge to double sided Sellotape on a coverslip. The samples were shadowed at angles between 15 and 45^{o} with gold and palladium using an Edwards Coating System E306A.

2.17.3 Immunogold labelling

The bacterial suspension was washed in PBS and 10 µl placed on a carbon coated grid and allowed to partially dry under a light bulb for 2-3 min. Excess fluid was removed using a strip of filter paper and the grids were immediately placed face down on a suitable dilution of antisera in 1%(w/v) bovine serum albumen (Sigma) and 1% (v/v) Tween 20 (Sigma) for 15 min. (See section 2.28). The grids were removed, washed thoroughly in 1%(w/v) bovine serum albumen and 1% (v/v) Tween 20 and placed on a drop of diluted (1:50) gold anti-rabbit conjugate for 15 min. The grids were washed thoroughly again and negatively stained with 1% (w/v) ammonium molybdate.

2.18 Removal of attached cells from the surfaces

The cells attached to the stainless steel discs were removed by placing the rinsed discs in PBS in a sonicating water bath. Four 15 sec sonication periods removed the majority of the cells from the discs (confirmed by direct epifluorescent microscopy- section 2.14), but did not affect the viability of the cells. In experiments comparing the properties of attached and liquid phase cells, the suspended cells were also exposed the same sonication regime.

2.19 Coulter Counter Analysis

Cell volume distributions were determined using a model ZBI Coulter Counter together with a Channelizer C100 connected to a BBC microcomputer. 100 µl of each sample was diluted in 20 ml Isoton (Coulter Electronics Ltd.) which had been prefiltered through a 0.22 µm Millipore filter. The profiles were obtained using a 30 µm orifice. The profiles were printed on a Tandy TRS-80 plotter. Latex particles of known size were used as calibration standards for cell volume analysis. When experimental conditions dictated, cells were fixed using 0.3% (v/v) glutaraldehyde in 0.02 M Tris for 15-20 min, washed and then diluted in Isoton. Cells treated in this way could be stored at 4°C for up to one week.

2.20 Isolation of bacterial outer membranes by detergent solubilization

Cells were grown in 100 ml volumes to the mid exponential or stationary phase and harvested by centrifugation at 10000g for 20 min. The pellet was resuspended in 10 ml 10 mM Tris-HCl (pH 8) and either stored at -20°C or the cells were broken by sonication (80S/50% cycle , 6 separate bursts of 15 sec with 1 min cooling periods, 20 kHz 6 µm peak to peak amplitude). The unbroken cells were removed by centrifugation at 4500g for 20 min at 4°C. The resulting supernatant was centrifuged at 48400 g for 60 min at 4°C. The pellet was resuspended in 150 µl distilled water and stored in 50 µl aliquots at - 20°C. When required 50 µl samples were thawed and extracted with 8 volumes (400 µl) of detergent solution containing 1.67% (w/v final concentration) of N-lauroylsarcosine and 11.1 mM (final concentration) Tris pH 7.6 at room temperature for 20 min. The insoluble outer membranes were pelleted at 48400 g for 90 min at 20°C. The pellet was resuspended in 50 µl of electrophoresis sample buffer (0.025M Tris pH 6.8, 8% (w/v) sodium dodecyl sulphate (SDS), 40% (v/v) glycerol, 20% (v/v) mercaptoethanol) for electrophoresis or 50 µl of distilled water for protein determination.

2.21 Soluble protein preparation

The supernatant produced after the 48400 g for 90 min centrifugation in section 2.19 contained soluble cell proteins.

2.22 Trypsin modification of the cell envelope

Cells were grown in 100 ml volumes to the mid exponential phase of growth and harvested by centrifugation at 9820 g for 20 min. The pellet was resuspended in 10 ml trypsin (1.0% (w/v) in PBS at pH 7.7) for 1 hr at 37°C after which the cells were washed twice in 10 mM Tris HCl (pH8) and finally resuspended in 10 ml 10 mM Tris HCl. Cells were also treated with heat inactivated trypsin in PBS (pH 7.7) and PBS (pH 7.7) as negative and untreated controls respectively.

2.23 Determination of protein concentration

Protein estimations were carried out using the Folin phenol reagent as described by Lowry *et al.* (1951) using bovine serum albumen (Sigma) as standard. The following solutions were prepared :-

A. 5% (w/v) sodium carbonate (Na_2CO_3)

B. 1% (w/v) copper sulphate ($CuSO_4$)

C. 2% (w/v) sodium potassium tartrate ($NaK(CHOHCOO)_2$)

D. Folin Ciocalteau reagent (Fisons) diluted 1:1 with distilled water

E. 1M sodium hydroxide (NaOH)

Samples were diluted in 0.5 ml distilled water in acid washed test tubes, then 0.5 ml of 1M NaOH was added and the samples boiled for 5 min and cooled. To 50 ml of solution A , 1 ml of solution B and 1 ml of solution C were added. 2.5 ml of this mixture was added to each sample and incubated for 10 min at room temperature. The samples were vortexed and 0.5 ml of solution D was added. After thorough vortexing the samples were allowed to stand at room temperature for 30 min. The absorbance of the samples was then measured at a wavelength of 750 nm against a reagent blank containing 0.5 ml of distilled water, and their

protein content determined by reference to a calibration graph of absorbance against bovine serum albumen concentration.

2.24 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

SDS-PAGE was performed using a discontinuous buffer system (Laemmli, 1970) including the ionic detergent sodium dodecyl sulphate (SDS) to dissociate proteins into their individual polypeptide subunits.

Both linear and two dimensional O'Farrell gels (O'Farrell, 1975) were used. The two dimensional gels were used when there was insufficient resolution of the protein bands in the linear gels.

2.24.1 Linear SDS-PAGE

The following stock solutions were prepared :-

Buffer A	0.75M Tris-HCl pH 8.8
	0.2% (w/v) SDS
Buffer B	0.25M Tris-HCl pH 6.8
	0.2% (w/v) SDS
Acrylamide Stock	44 g Acrylamide
	0.8 g Bisacrylamide
	100 ml distilled water
Ammonium persulphate (AMPS)	1% (w/v) AMPS made immediately before use.
Reservoir buffer	0.25M Tris
	0.129M glycine
	0.1% (w/v) SDS

Gels with various acrylamide concentrations were used depending upon the particular samples. Table 2.2 shows the volumes of the above stock solutions required to give a range

of acrylamide concentrations for the resolving gel, similarly Table 2.3 shows the volumes required for a range of stacking gels.

Table 2.2

Volumes of stock solutions required to give various concentrations of acrylamide in the resolving gel.

Stock solution	Volume of stock solution (ml) to give acrylamide concentrations of ;			
	15%	**12%**	**11%**	**8.5%**
Buffer A	27.0	27.0	27.0	27.0
Acrylamide stock	18.4	14.8	16.6	10.6
Distilled water	7.2	10.8	12.0	15.0
AMPS	1.9	1.9	1.9	1.9
TEMED	100 µl	100 µl	130 µl	130 µl

TEMED - NNN' tetramethylethylenediamine

Gels were cast between glass plates (18x16 cm) with a stacking gel on top of the resolving gel. Glass plates were separated by lucite spacers and the plates clamped together. Approximately 25 ml of resolving gel mixture was poured between the glass plates using a pipette to run the liquid down one side of the gel to avoid trapping air bubbles. The gel mixture was overlaid with water saturated butanol and allowed to polymerise. When set the butanol was washed off using distilled water and the stacking gel poured on top of the resolving gel with a lucite slot former pushed into the top of the stacking gel.

After the stacking gel had polymerised the slot former was removed and the gel placed in the electrophoresis tank containing reservoir buffer ready for loading. Care was taken to ensure that air bubbles were not trapped between the glass plates at the bottom of the gel. The upper reservoir was fastened into position and partially filled with reservoir buffer. The samples were then loaded (see section 2.23.3 for sample preparation) before filling the upper reservoir completely.

The gel was run at 8 mA for 16 hr or 30 mA for 3 hr until the bromophenol blue dye front reached the bottom of the gel. Gels were removed and stained accordingly.

Table 2.3

Volumes of stock solutions required to give various concentrations of acrylamide in the stacking gel.

Stock solution	Volume of stock solution (ml) to give acrylamide concentrations of ;		
	7.5%	4.5%	2.5%
Buffer B	10.0	10.0	10.0
Acrylamide stock	3.3	3.0	1.2
Distilled water	6.7	7.0	8.8
AMPS	0.5	0.5	0.5
TEMED	40 µl	40 µl	40 µl

2.24.2 Two dimensional O'Farrell gels

In this gel system proteins were separated by their isoelectric points in the first dimension and according to molecular weight in the second dimension to obtain maximal resolution of proteins in complex mixtures (O'Farrell, 1975).

A stable pH gradient was formed using commercial ampholines and the proteins electrophoresed until their net charge was zero and therefore migration ceases. However as the original method of O'Farrell (1975) does not allow for the effects of detergent solubilization on the charge distribution of the protein, the modified method of Ames and Nikado (1976) was used. This involves membrane solubilization with SDS and then first dimension electrophoresis in the presence of the non ionic detergent Nonidet P-40 (NP-40) and urea to remove the SDS bound to the protein.

The following solutions were prepared;

(i) 30% (w/v) acrylamide stock for isoelectric focusing

Acrylamide	28.38 g
Bisacrylamide	1.62 g
Distilled water to	100 ml

(ii) Isoelectric focusing gel mixture (sufficient for 10 tubes) containing

Urea	5.5 g
30% Acrylamide stock	1.33 ml
10% (w/v) NP40	2.0 ml
Distilled water	1.97 ml
Ampholines pH 5-7	0.4 ml
Ampholines pH 3.5 -10	0.1 ml

Warm to dissolve, add 7 µl TEMED and 10 µl 10% (w/v) AMPS

(iii) Sample dilution buffer containing

Urea	5.5 g
Ampholines pH 5-7	0.4 ml
Ampholines pH 3.5 - 10	0.1 ml
Mercaptoethanol	0.5 ml
NP-40	0.8 ml
Distilled water to	10.0 ml

(iv) Laemmli sample buffer

Tris-HCl pH 6.8	62.5 mM
SDS (w/v)	2 %
Mercaptoethanol (v/v)	5 %
Glycerol (v/v)	10 %
Bromophenol blue (w/v)	0.5 %

The isoelectric focusing gels were made in 150 mm length x 4 mm internal diameter tubes which had been cleaned by boiling in 0.1M HCl for 30 min, followed by thorough rinsing in distilled water and a final rinse of ethanol. The bottom of the tubes were covered with two layers of parafilm and the gel mixture added using a long form pasteur pipette to give a length of 10 cm. This was performed in a constant temperature room (37^{o}C) to prevent crystallisation of the urea and the gel setting too rapidly. The gel was overlaid with 8M urea and allowed to set for 1-2 hr. The urea overlay was removed and replaced with 20 µl of sample dilution buffer which was overlaid with distilled water. After 2 hr the water and buffer were removed and a fresh 20 µl of sample dilution buffer was added and overlaid with 0.02M NaOH.

The parafilm was carefully removed and the tubes were pushed into the holder, which was lowered into the electrophoresis tank (containing 0.01M phosphoric acid) at an angle to prevent air bubbles being trapped at the bottom of the tubes. The upper reservoir was added and filled with 0.02M NaOH. The tube gels were pre run at 200V for 15 min, 300V for 30

min and then 400 V for 30 min to form the isoelectric gradient. The sodium hydroxide and sample dilution buffer were removed from the upper reservoir and the tubes. The samples (see section 2.23.3.2 for sample preparation) were loaded and overlaid with 10 µl of a solution containing 5M urea, 0.4% (v/v) Ampholines pH 5-7 and 0.1% (v/v) Ampholines pH 3.5-10. The remainder of the tubes and the reservoir were refilled with 0.02M NaOH and the proteins focused by electrophoresis at 400V for 16 hr followed by 800V for 1 hr.

After electrophoresis gels were extruded from the tubes by pressure from a water filled syringe into a screw topped tube containing 5 ml Laemmli sample buffer and allowed to equilibrate for 1 hr after which the gels were immediately loaded onto the second dimension or stored frozen at -20 ^{o}C.

The second dimension consisted of linear gels prepared as described in 2.23.1 except that the slot former was not used and the stacking gel was also overlaid with water saturated butanol. The stacking gel was poured to 0.5 cm below the top of the gel plates. Once the stacking gel had set the butanol was washed off with distilled water. The space at the top of the gel was filled with hot 1.0% (w/v) agarose in Laemmli sample buffer plus bromophenol blue. The tube gel was carefully placed on top of the agarose and gently pushed into the agarose to ensure that no air bubbles were trapped. Agarose was used to seal the tube gel into place. A small well was made at one end of the tube gel for the molecular weight standards.

The gel was run at 14 mA for 16 hr at room temperature and then removed, fixed and stained accordingly.

2.24.3 Preparation of samples

2.24.3.1 Linear SDS-PAGE

The amount of protein sample loaded was adjusted depending upon the protein concentration of the samples. For gels stained by Coomassie blue and for Western blot analysis 50-100 µg of protein was loaded per track, whilst for the more sensitive silver stain 5-10 µg of protein was loaded.

Samples were denatured by adding electrophoresis sample buffer (0.025M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 0.5% (w/v) bromophenol blue) and boiling for 5 min. Denatured samples were loaded into the wells in the linear gel using a Hamilton glass syringe (either 0.1 ml or 0.01 ml) which was thoroughly rinsed between samples.

Protein molecular weight of size range 14.4 - 94.0 KDa (Pharmacia) were reconstituted according to the manufacturers instructions. Bromophenol blue was added and 3 µl routinely run on SDS-PAGE.

2.24.3.2 Two dimensional Isoelectric focusing gels

Due to problems with smearing in the second dimension it was necessary to use an acetone precipitation step to remove lipids from the various samples. The pellet obtained at the end of the outer membrane preparation was resuspended in 100 µl of acetone at -20°C instead of electrophoresis buffer. This was microfuged for 5 min and the pellet dried under a stream of nitrogen and resuspended in Laemmli sample dilution buffer without tracker dye and solubilized by heating at 75°C for 2 min. Two volumes of sample dilution buffer were added to the SDS solubilized samples to remove SDS bound to protein. These samples were kept frozen at -20°C until use.

2.25 Lipopolysaccharide (LPS) extraction

The method described by Hitchcock and Brown (1983) was used. Outer membrane sample (prepared as described in section 2.19) at 2 mg ml^{-1} was added to an equal volume of 2x sample buffer (0.25M Tris-HCl pH 8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (v/v) mercaptoethanol, 0.005% (w/v) bromophenol blue). After boiling for 5 min, 25 µg of Proteinase K (Sigma) was added to 100 µg of protein by addition of the appropriate volume of a stock solution of Proteinase K at 2.5 mg/ml in 1x sample buffer. The sample was heated at 60°C for 1 hr and stored at -20°C until use.

2.26 SDS-PAGE of lipopolysaccharide

The lipopolysaccharide (LPS) samples were separated using linear polyacrylamide gels prepared as described in section 2.23.1 except 4M urea was added to the 15% resolving gel. Aliquots of sample were electrophoresed at 30 mA for 3 hr. Smooth LPS from *Escherichia coli* O111: B4 (Sigma) was used as a control, routinely 20 µl of a 10 mg ml^{-1} solution in 1x sample buffer was used.

2.27 Staining of polyacrylamide gels

2.27.1 Coomassie blue stain

Gels were immersed in the staining solution (45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) coomassie blue R250) for 3-4 hr and then destained in 45% (v/v) methanol with 10% (v/v) glacial acetic acid until background colouration was at a minimum.

2.27.2 Silver stain for protein

Silver stain is approximately 10-20 times more sensitive than coomassie blue staining, requiring a minimum of approximately 5-10 ug protein per track. The method of Wray *et al.* (1981) was used. Gels were soaked in 50% (v/v) methanol for a minimum of 8 hr with three changes. 1.6 g of silver nitrate was dissolved in 8 ml of distilled water and slowly added with shaking to a solution containing 42 ml of 0.36% (w/v) NaOH and 2.5 ml of ammonia solution. The volume was made up to 200 ml with distilled water and then used to stain the gel for 15 min. After 2 washes in distilled water for 5 min each the gel was soaked in developer (2.5 ml 1% (w/v) citric acid, 0.4 ml formaldehyde made up to 500 ml with distilled water) until the bands appeared. The reaction was stopped by soaking the gel in a solution containing 10% (w/v) glacial; acetic acid and 45% (v/v) methanol.

2.27.3 Silver stain for LPS

The method described by Hitchcock and Brown (1985) was used. The gel was soaked overnight in 200 ml 25% (v/v) isopropanol in 7% (v/v) glacial acetic acid and then oxidised

for 5 min in 150 ml distilled water with 1.05 g periodic acid and 4 ml 25% (v/v) isopropanol in 7% (v/v) glacial acetic acid (made up immediately before use). After eight 30 min washes, each with 200 ml distilled water the gel was silver stained for 10 min in a solution containing 28 ml 0.1M NaOH, 1 ml concentrated (29.4%) ammonium hydroxide, 5 ml 20% (w/v) silver nitrate and 115 ml distilled water (made up just before use with constant stirring). The gel was washed again for four 10 min washes in 200 ml distilled water and the soaked in developer which contained 50 mg citric acid and 0.5 ml formaldehyde in 1000 ml distilled water, made up just before use at an optimum temperature of 25°C since below this temperature staining of protein as well as LPS occurs. Once the bands had developed sufficiently (takes 10-20 min) the gel was soaked in a stop solution containing 200 ml distilled water and 10% (v/v) glacial acetic acid for 1 hr. After a final wash in 200 ml distilled water the gel was stored in an airtight container with a small amount of water to prevent desiccation.

2.28 Photography of gels

Stained gels were routinely photographed using an Olympus camera with Kodak Tmax 100 (ASA 100) film.

2.29 Production of antisera

Each rabbit (Sandy half lops) was bled before exposure to the organisms to ensure that the antibodies raised were specific, this was designated as the control (zero) blood. The blood was stored overnight at 4°C and the serum separated from the blood clot by centrifugation at 2000 g for 15 min at 4°C. Serum was stored in 1 ml aliquots at -20°C.

Exponential phase cells of *S.liquefaciens, S.cohnii* and *P.fragi* were washed twice in PBS and inactivated by formalin treatment (0.5% (v/v) formaldehyde in PBS for 18 hr). Inactivated cells, at 10^8 cells ml^{-1}, were stored in 1 ml aliquots at minus 20°C. 1 ml aliquots of inactivated cells were injected intravenously into the marginal ear vein of the rabbits. This was repeated weekly for a month after which the rabbits were bled once a week for 4-5 weeks and the sera collected as described above.

2.30 Rocket Immonoelectrophoresis

The presence of antibodies in the sera was checked qualitatively by rocket immunoelectrophoresis. Immuno plates were prepared using 1% (w/v) agarose in barbitol buffer (50 mM sodium barbitol, 10 mM barbituric acid, pH 8.6) containing 0.5,1.0 or 5.0 % (v/v) crude antisera. Antigen was solubilized with a final concentration of 1% (w/v) Triton x-100. 20 µl of sample was loaded in 2 mm diameter wells and electrophoresed at 8 mA for 16 hr at room temperature. Immuno plates were washed twice in PBS, once in distilled water with partial drying between washes. Finally the plates were dried in a hot air oven before staining with coomassie blue for 10 min.

2.31 Passive Haemagglutination

Haemagglutination was used to determine quantitatively the antibody titre. The antigen for the haemagglutination was obtained by saline extraction. Confluent growth on four agar plates was resuspended in 4 ml of saline and boiled for 1 hr. This was centrifuged at 4000 g for 10 min and the resulting supernatant contained the soluble antigens. This preparation was diluted 1:10 in PBS, pH 7, and incubated at 37°C for 1 hr with an equal volume of 1% washed sheep red blood cells (SRBC). The suspension was washed three times in PBS and finally resuspended in PBS to give 0.5% SRBC. Doubling dilutions of antisera were made in a multiwell plate with round bottomed wells so that each well contained 25 µl. To each well 25 µl of SRBC suspension was added and the plate gently shaken. After incubation at 37°C for 1 hr the plate was stored at 4°C overnight . The plate was examined for agglutination and the highest dilution showing agglutination corresponded to the antibody titre.

2.32 Western Blotting

2.32.1 Transfer of proteins to nitrocellulose

Proteins were separated by denaturing polyacrylamide gels (SDS-PAGE section 2.24.1). After electrophoresis the stacking gel was removed and the gel was soaked in

Western Transfer Buffer (WTB ; 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol) for 10-15 min. A nitrocellulose filter was cut to the size of the gel, soaked in WTB and carefully placed on top of the gel ensuring that all the air bubbles had been removed. The gel and filter were placed between 4 pieces of filter paper soaked in WTB and sandwiched in the holder and placed in the Biorad Transblot tank containing WTB. The proteins were transferred to the nitrocellulose at 40 V for 3 hr at 4°C. After transfer the nitrocellulose was stained to visualise the protein bands by washing for 10 min in Ponceau S (0.5% (w/v) in 5% (v/v) TCA). The position of the slots and protein markers was noted and the stain was removed by washing the filter in 20 ml PBS for 10 min. The filter was then washed in 10 ml PBS containing 2% (w/v) dry milk (Marvel) for a minimum of 1 hr to allow protein to bind non specifically to the nitrocellulose. The PBS/dry milk was replaced with a fresh 10 ml volume of PBS with 2% (w/v) dry milk containing primary antibody (crude sera) at an appropriate dilution (1:200 for *S.liquefaciens* and *P.fragi* and 1:50 for *S.cohnii*) and incubated overnight at room temperature with gentle shaking. The filter was then washed three times for 10 min each time in 10 ml PBS containing 0.1% (v/v) Tween 20.

2.32.2 Detection of the antigen-antibody complexes using a horse radish peroxidase colour reaction.

Peroxidase conjugated goat anti-rabbit IgG (Sigma) was used as the secondary antibody and 4-chloro-1-naphthol (Sigma) as the colour reagent. After the washes in PBS with 0.1% (v/v) Tween 20, the filter was transferred to fresh PBS with 0.1% (v/v) Tween 20 (10 ml) containing a 1:300 dilution of secondary antibody and incubated at room temperature for 1-2 hr with gentle shaking. After two 10 min washes in 10 ml PBS with 0.1% (v/v) Tween 20 and two washes in 10 ml PBS , the filter was transferred to the staining solution. This solution consisted of 0.06% (w/v) 4-chloro-1-naphthol in 20% (v/v) methanol which was mixed to an equal volume of a solution containing 3% (w/v) NaCl, 2% (v/v) 1M Tris-HCl pH 7.5, 0.1% (v/v) hydrogen peroxide just before use. Before the reaction reached completion the filter was washed in distilled water (3 changes over 30 min), air dried, photographed and stored in the dark.

2.33 Exopolysaccharide extraction

Methods 1 and 2 were simple and relied upon physical separation only. Contamination with intracellular and cell bound components were kept to a minimum. In certain cases the lyophilised material produced by either method was purified by extraction with 45% (w/v) aqueous phenol (method 3).

2.33.1 Method 1 : Crude exopolysaccharide extraction (Ha *et al.*, 1991)

Cells were grown in 500 ml volumes in 2 l flasks and harvested by centrifugation at 10000 g for 1 hr at 4°C. The supernatant was dialysed against 15 volumes of distilled waster with three changes over 3 days at 4°C. The dialysates were re centrifuged to remove precipitated protein and other insoluble material and the resulting supernatant freeze dried and stored at - 20°C.

2.33.2 Method 2 : Crude exopolysaccharide extraction (Beech *et al.*, 1991)

Cells were grown in 500 ml volumes in 2 l flasks and harvested by centrifugation at 10000 g for 30 min at 4°C. Three volumes of isopropanol were added to the supernatant and left for 48 hr at 4°C. The precipitated polymer was collected by centrifugation at 10000 g for 20 min at 4°C, dissolved in 10 ml distilled water and dialysed overnight at 4°C. The dialysate was freeze dried and stored at - 20°C.

2.33.3 Method 3 : Partial purification of crude exopolysaccharide (Lambert, 1988)

Lyophilized exopolysaccharide was obtained as described in section 2.32.2. and dissolved in 0.25 M NaCl at 10 mg ml^{-1}. This was added to a half volume of 0.25 M NaCl containing 4% (w/v) cetyltrimethylammonium bromide (CTAB) for 15 min at room temperature. The precipitated RNA-CTAB complex was removed by centrifugation at 10000 g for 1 hr. Distilled water was slowly added to the supernatant until a second precipitate formed (approximately three volumes were required). The mixture was allowed to stand in an ice bath for 2 hr and the precipitated exopolysaccharide was collected by centrifugation. The precipitate was dissolved in a minimum volume of 1 M NaCl (5-8 ml

for 500 mg of starting material) and the acidic polysaccharide was precipitated again by the addition of 8 volumes of ethanol. The mixture was allowed to stand in an ice bath for 2 hr and the precipitate collected by centrifugation. The 1 M NaCl / ethanol extraction was repeated and the final precipitate was dissolved in a minimum of distilled water and lyophilised.

2.34 Total sugar assay

The method of Dubois *et al.* (1956) was used. To 2 ml of sugar solution containing approximately 10-70 μg of sugar, 50 μl of 80% (w/v) phenol was added. Concentrated sulphuric acid (5 ml) was added rapidly and directed at the liquid surface. The samples were allowed to stand for 10 min, vortexed and then placed in a water bath at 30 ^{o}C for 10-20 min. The colour which developed was stable for several hours. The absorbance of the samples was measured against a reagent blank containing 2 ml distilled water at wavelengths of 490 nm for hexoses and 480 nm for pentoses and uronic acids. Their sugar content was determined by reference to a calibration graph of absorbance against glucose concentration over the range 0-100 μg ml^{-1}.

2.35 Uronic acids assay

The method described by Blumenkrantz and Absoe-Hansen (1973) was used to measure uronic acids in polysasccharides without prior degradation as well as free sugars. Aqueous samples (0.6 ml) containing up to 50 μg of uronic acids were vigorously mixed with 3.6 ml of 0.0125M disodium tetraborate in concentrated sulphuric acid and incubated in a boiling water bath for 5 min. After cooling in ice, 60 μl of 0.15% (w/v) m-phenyl phenol in 0.5% (w/v) sodium hydroxide was added. Exactly 5 min after this addition the absorbance was read at 520 nm against a reagent blank containing 0.6 ml of distilled water. The uronic acid concentration was determined by reference to a calibration graph of absorbance against glucuronic acid concentration where 10 μg of glucuronic acid gives an absorbance of approximately 0.25.

2.36 Thin layer chromatography separation of neutral sugars

To 1 mg of freeze dried exopolysaccharide, 100 µl of 0.5M sulphuric acid was added and the mixture vortexed and then hydrolysed at 100°C for 4 hr. The hydolysate was neutralised with a saturated solution of barium hydroxide by removing small samples and blotting onto universal paper. To remove the barium sulphate formed by neutralisation the sample was centrifuged at 3000 g for 15 min and the supernatant removed and lyophilised. 10 µl of distilled water was added to resuspend the hydrolysates and the samples loaded onto cellulose glass plates by spotting successive multiple spots in 2 µl aliquots. The following sugar standards were used (3 µl of 10 mg ml^{-1} solutions) :- glucose, rhamnose, mannose, galactose, ribose, and fucose.

The plate was placed into a tank containing n butanol:pyridine:water (6:4:3) which had been stored in the tank for 24 hr to saturate the atmosphere. The plate was run until the solvent front was 1 cm from the top, the plate was removed and allowed to dry in the fume hood and then returned to the tank and rerun in the same direction to give better resolution of the sugars. The plate was then dried and stained by spraying with anilium hydrogen phthalate (0.93 g aniline, 1.6 g 0-phthalic acid in 100 ml n-butanol saturated water). The sugars were observed after baking the plate at 100°C for 10-15 min.

2.37 Exopolysaccharide staining for light microscopy

The method described by Allison and Sutherland (1984) was used to stain the exopolysaccharide around the cells. As the light microscope was used only glass surfaces were applicable, so biofilms grown on glass coverslips (13 mm diameter) rather than stainless steel surfaces were used (as described in section 2.12). The surfaces were removed from the fermenter system at intervals, rinsed in PBS and submerged in 10 mM cetyl pyridium chloride and air dried for 20-30 min. The quaternary ammonium salt precipitates the polysaccharide. The cells were fixed by gentle heating and allowed to cool. The polysaccharide was stained with a 2:1 mixture of saturated aqueous congo red and 10% (v/v) Tween 80 solution - the latter intensifies the congo red staining. After rinsing carefully the cells were stained with 10% (v/v) Ziehl carbol fuschin, rinsed again and then air dried at

37°C. The coverslips were attached to glass slides using double sided Sellotape and examined under the light microscope.

2.38 Hydrophobicity Assays

2.38.1 Bacterial adhesion to hexadecane (BATH)

Cells were harvested by centrifugation and washed twice in PUM buffer (22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4.7H_2O$, 1000 ml distilled water, pH 7.1) and finally resuspended in PUM buffer to give an absorbance at a wavelength of 400 nm of approximately 1.5 (Rosenberg *et al.*, 1980). To 1.2 ml of this suspension was added 0.1 ml of n-hexadecane (Sigma) and incubated for 10 min at 30°C. The sample was then vortexed for 120 sec, left to stand for 15 min to allow the two phases to separate and the absorbance at 400 nm of the lower aqueous phase was measured. Comparison of this absorbance with that of the original suspension allowed the adherence to the hexadecane to be calculated as shown below :-

$$\text{Adhesion to hexadecane} = \frac{(\text{Original } A_{400nm} - A_{400nm}) \times 100}{\text{Original } A_{400nm}}$$

2.38.2 Hydrophobic Interaction Chromatography (HIC)

This method (Smyth *et al.*, 1978) also measures the adhesion of the cells to a hydrocarbon phase. The cells were harvested by centrifugation, washed twice in 1M NaCl pH 7 and resuspended in 1M NaCl pH 7. Glass wool plugged pasteur pipettes were washed twice with 95% (v/v) ethanol followed by 1M NaCl pH 7. Phenyl sepharose CL4B (Sigma) was diluted 1:1 with 1M NaCl pH 7 and 0.6 ml was added to each pipette. The absorbance at 600 nm of the original sample was compared to that of the eluate and the percentage retention by the hydrophobic phenyl sepharose calculated. Unsubstituted sepharose (Sigma) was used as a control to ensure that mechanical entrapment in the gel matrix did not occur.

2.39 Electrostatic interaction chromatography

This method (Pederson, 1980) measures the cell adhesion to anion and cation exchange resins. The cells were harvested, washed and resuspended in 0.1M NaCl pH 7. Glass wool plugged pasteur pipettes were washed with 95% (v/v) ethanol followed by 0.1M NaCl pH 7. The ion exchange resins were diluted 1:1 with 0.1M NaCl pH 7. 1 ml aliquots of diluted anion exchange resin (Dowex 2 strongly basic anion exchanger with 8% cross linkage and 100 - 200 dry mesh, ionic form chloride) and cation exchange resin (Dowex 50W strongly acidic cation exchanger with 8% mesh size and 100 - 200 dry mesh, ionic form hydrogen) were added to the pipettes. The columns were washed with 0.1M NaCl pH 7. The absorbance at 600 nm of the original cell suspension was compared to that of the eluates and the percentage retention by the anion and cation exchange resins calculated. From these percentages the ratio of retention by the anion to cation exchange resin was calculated as follows :-

$$\text{Ratio of retention by anion:cation} = \frac{\%\ \text{Retention by anion exchange resin}}{\%\ \text{Retention by cation exchange resin}}$$

2.40 Determination of microbial activity

An estimate of microbial activity may be obtained by measuring the incorporation of ^{3}H thymidine into DNA (Jeffery and Paul, 1986). Tritiated thymidine (1 ml) at 0.5 µCi and 0.1 µmole thymidine was added to either a 2 ml sample of liquid phase chemostat cells or to a stainless steel disc and attached biofilm (the volume was adjusted to 2 ml with media). Triplicate samples were routinely used and sterile stainless steel discs were used to determine the adsorbance of thymidine to the surface. The samples were incubated at 20°C for 2-4 hr . Following incubation 100 µl of 1 mg ml^{-1} DNA was added to increase the extraction yield, followed by the addition of 1 ml 1 M NaOH. The samples were incubated at 100°C for 1 hr, centrifuged and two 1 ml samples of supernatant were collected. To each 1 ml sample 5 ml cold 10% (w/v) trichloroacetic acid (TCA) was added, one sample was retained on ice and the second digested at 100°C for 1 hr. The two fractions were filtered through 0.2 µm

membranes, washed with 10% (w/v) TCA, dried and 5 ml LKB Optiphase Safe scintillation fluid was added and the samples counted in a LKB 1219 Rackbeta liquid scintillation counter. To enable a comparison between liquid phase and attached cells it was necessary to determine the concentration of cells in each phase. The liquid phase cell concentration was determined by serial dilution and spread plating onto nutrient agar. The attached population was enumerated by acridine orange staining and epifluorescent microscopy as described in section 2.14. Thymidine incorporation per cell per hour was calculated.

2.41 Measurement of glucose respiration

A 2 ml sample of liquid phase cells or a stainless steel disc with 2 ml of medium was added to a flask and 0.4 ml 2.5 M NaOH carefully added to the central well. Labelled glucose solution (1 ml) containing 1 μCi of ^{14}C glucose and 10 mM glucose was added to the main compartment and the flask sealed with a subaseal. The flasks were incubated at 20°C for 4-6 hr after which 2 ml 50% (v/v) sulphuric acid was carefully injected. The flasks were allowed to shake for a further 20 min during which the carbon dioxide was absorbed by the alkali in the inner well. The radioactivity in the well was counted by liquid scintillation by transferring 100 μl to a scintillation vial, followed by the addition of 5 ml scintillation fluid. The potential respiration of glucose per cell per hour was calculated in order to allow comparison of rates of attached and liquid phase populations.

2.42 Statistical analysis

Where appropriate the data was analysed statistically using the Analysis of Variance options of the Minitab computer statistical package. Results described as significantly different in the results chapters were significantly at the 0.05 significance level.

CHAPTER THREE

3 ISOLATION AND CHARACTERIZATION OF ORGANISMS FOUND ON SURFACES IN FOOD PROCESSING ENVIRONMENTS

3.1 Introduction

Of major importance in food processing is the freedom of microbial and foreign body contamination from the finished product. Such contamination may lead to product recalls with the associated adverse publicity resulting in the loss of customers, sales and profit (Holah and Kearney, 1992).

Contamination of the product may arise from four main sources :- the constituent raw materials, surfaces, people (and other animals) and the air. Food may pick up contamination from the product contact surfaces directly, or from the food handlers. The handlers may also transfer contamination from non-food contact surfaces such as walls and doors. The air acts as a source of contamination by transporting microbes and particles from outside the production area, and also may carry contamination from non-product contact surfaces to contact surfaces. Cleaning systems, such as high pressure hoses, have been shown to generate aerosols of sufficient size to carry bacteria onto production lines several metres away (Holah *et al.*, 1990).

In food processing, production runs may be from a few hours to several days. The cleaning regimes are co-ordinated to coincide with the production intervals. Consequently the time available for microbial attachment and proliferation on the surface, particularly food contact surfaces, may be relatively short. However, environmental surfaces such as walls and ceilings are generally cleaned less frequently and therefore the niches for more extensive biofilm development do exist in the factory environments.

The type of microbial species present is important as relatively high levels of non-spoilage or non-pathogenic micro-organisms on the surfaces may be tolerated, but the presence of pathogens such as *Salmonella* and *Listeria* species would not generally be acceptable. The presence of non-spoilage, non-pathogenic organisms in a biofilm has been shown to protect *Listeria* species from disinfectant action (Frank, 1990).

The irregular inputs of nutrients and /or stresses due to desiccation, cleaning and disinfection mean that some bacteria are likely to be in a quiescent non-culturable form. The proportion of culturable cells in the food processing environment is unknown, but in most natural environments only a small percentage (0.1-1.0%) of the viable microbial population is culturable (Bianchi and Bianchi, 1991). Work characterizing the 16S ribosomal sequences from naturally occurring biomasses has shown that the bacterial populations are more diverse than originally thought due to the presence of unculturable organisms (Olsen, 1990).

Biofilm development in food processing environments can have detrimental effects on the microbial status of the food resulting in an increased chance of food spoilage affecting shelf life and increasing the risk of food poisoning.

Bouman *et al.* (1982/4) examined the levels of bacteria present on surfaces exposed to raw and pasteurized milk and found 10^4 and 10^6 cells cm^{-2} respectively after 12 hr. Lewis and Gilmour (1987) examined stainless steel and rubber exposed to milk for 5 days and found between $8.13x10^3$ and $8.55x10^4$ cells cm^{-2}.

Holah and co-workers (1989 and 1992) attached stainless steel plates to a wide variety of product type environments including fish filleting, butter milk production, and baked bean production. They found a range of levels of bacteria to a maximum of 10^7 cells cm^{-2} in the baked bean production. The levels of bacteria were enumerated by direct epifluorescent microscopy and are therefore the counts were total cell counts including non viable or non culturable cells.

Bacteria will attach to and grow on a wide variety of surface types including stainless steel, aluminium, enamelled steel, high density nylon, polypropylene, PVC, polycarbonate, Teflon and mineral resin (Holah and Thorpe, 1990). Within four hours all of these surfaces with the exception of teflon had an attached population in excess of $5x10^7$ cells cm^{-2}.

Sanitation procedures must therefore reduce these numbers to satisfactory levels. Of primary importance in the cleaning programme is the removal of product soil by detergents, and this step alone significantly reduces the attached population. However viable bacteria are likely to remain on the surface and require disinfection. The commonly used disinfectants include chlorine releasing compounds, quaternary ammonium compounds, amphoterics,

iodine compounds and peracetic acid. Chlorine, in particular, is inactivated by organic matter and therefore is used once the product soil has been removed.

The efficiency of the sanitation programme is dependant on the hygienic design of the processing equipment and environment. The most commonly used material in food processing environments is stainless steel due to its strength, corrosion and abrasion resistance, malleability and cleanability (Timperley *et al.*, 1992). Equipment that is hygienically designed prevents retention of product out of the main product flow and is readily cleaned. Poor hygienic design is often characterized by rough surfaces, crevices and dead spaces where product and micro-organisms may be retained, and are extremely difficult to remove.

The studies described above examined the levels of the bacteria on the surfaces in different processing environments. This study evaluated the nature of the bacteria present as well as the levels of bacteria present on the surfaces.

Three sites were sampled including a diverse range of product type environments. The first site was a large supermarket which comprised three very distinct areas (i) the staff canteen kitchen, (ii) preparation of raw and cooked meats and (iii) fish preparation. These areas were spatially isolated, with dedicated staff for each area. Site two was a smoked salmon factory with a low risk fish preparation area and a high risk operation which involved slicing and packaging of the smoked product. The third site was a pig abattoir.

3.2 Aims

The objective was to determine the types of bacteria present in food processing environments and their relative concentrations to enable selection of a combination of bacteria from the same environment to be used to model biofilm development in the laboratory.

As organisms rapidly become attenuated by prolonged laboratory culture it was considered important that fresh isolates were used. The aim was also to determine the morphological, biochemical and basic growth characteristics of the selected strains.

3.3 Results and discussion

3.3.1 Food environment survey

3.3.1.1 Supermarket

Three distinct areas were sampled (i) the staff canteen preparation area (ii) the meat preparation area and (iii) the fish preparation area. Table 3.1 shows the viable cell counts per cm^2 at each sample site.

The canteen stainless steel table used for the preparation of vegetables was in use at the time of sampling and may explain the relatively high count obtained ($>2.50x10^3$ cells cm^{-2}). The other stainless steel table was used for the preparation of cooked meats and pastries and therefore did not come into contact with raw products so the lower count obtained was to be expected. Bacteria were not detected on the canteen floor and this may be attributable to the use of stronger biocides on this surface.

The knife handle in the meat preparation area had a relatively high count, and the stainless steel table was slightly less populated. Both these surfaces had been recently cleaned.

The sink in the fish preparation area had very few bacteria on the surface, but the waste disposal system had a significantly higher level of contamination. Obviously this system is used for the disposal of innards etc. which will by their nature have high levels of bacteria. Although this surface is not a product contact surface it may still be a source of contamination by transfer via personnel.

Table 3.1

Viable counts per cm^2 on the surfaces sampled in the supermarket.

Sample site description	Total	Coliforms	Fastidious
Canteen kitchen area			
Canteen SS table (meat and pastries)	$4.47x10^1$	$1.38x10^1$	$2.75x10^1$
Canteen SS table (vegetables)	$>2.50x10^3$	$>2.50x10^3$	$>2.50x10^3$
Canteen floor	$<2.50x10^0$	$<2.50x10^0$	$<2.50x10^0$
Meat preparation area			
Delicatessen SS table	$2.50x10^0$	$<2.50x10^0$	$<2.50x10^0$
Knife handle in meat preparation area	$>2.50x10^3$	$4.95x10^2$	$>2.50x10^3$
SS table in meat preparation area	$2.71x10^2$	$7.50x10^0$	$3.00x10^1$
Tin opener	$2.50x10^0$	$<2.50x10^0$	$3.75x10^0$
Fish preparation area			
SS sink in fish preparation area	$<2.50x10^0$	$2.50x10^0$	$2.50x10^0$
Waste disposal in fish preparation area	$3.99x10^2$	$9.38x10^1$	$6.45x10^2$

SS - Stainless steel

$<2.50x10^0$ limit of detection

The characterization results show that a wide range of bacteria were present on the various surfaces (Table 3.2). Most of the bacteria isolated are common environmental organisms e.g. *Citrobacter freundii, Staphylococcus sciuri, Enterobacter agglomerans* and *Acetobacter calcoaceticus* are commonly found in nature. However the presence of the human commensal *Staphylococcus,* showed that products were being contaminated by the handlers.

The various bacterial biofilm communities were naturally very heterogeneous e.g. the range of bacteria found on the canteen stainless steel table (5 species), on the fish area waste disposal system (7 species) and particularly on the knife handle where twelve different bacterial species were found. Alternatively, the bacterial biofilm was relatively homogeneous e.g. a single species *(Staphylococcus xylosus)* was isolated from the tin opener and a single genus was present on the stainless steel delicatessen table.

The bacteria isolated from the three areas (canteen, meat and fish) were quite distinct, and reflected the spatial isolation of the three areas as well as the limited movement of staff between the three areas. *Providencia* and *Citrobacter* species were unique to the fish preparation area, whilst *Klebsiella* and *Acetobacter* species were unique to the canteen area and *Serratia* species were only found in the meat preparation area.

Table 3.2

Bacteria isolated from the surfaces in the supermarket.

Sample site description	Organisms identified
Canteen kitchen area	
Canteen SS table (meat and pastries)	*Acetobacter calcoaceticus var anitraeus* *Klebsiella pneumoniae* Unidentified Gram negative cocci and rods, Gram positive cocci
Canteen SS table (vegetables)	*Klebsiella pneumoniae* *Staphylococcus* sp. Unidentified Gram negative rods
Meat preparation area	
Knife handle in meat preparation	*Serratia marsescens* *Staphylococcus auricularis* *Streptococcus cremoris* *Streptococcus thermophilus* *Flavobacterium* sp. *Serratia liquefaciens* *Enterobacter agglomerans* *Rahnella aquatilis* *Serratia plymithica* *Serratia* sp. *Pseudomonas* sp. Unidentified Gram negative rod
Delicatessen SS table	*Staphylococcus xylosus* *Staphylococcus* sp.
Meat prep. area SS table	*Staphylococcus cohnii* *Staphylococcus lentus* *Staphylococcus sciuri* *Enterobacter agglomerans*
Tin opener	*Staphylococcus xylosus*
Fish preparation area	
Waste disposal in fish area	Several *Psudomonas sp.* *Providencia alcalfaciens* *Providencia stuartis* *Citrobacter freundii* *Pseudomonas aeruginosa* *Staphylococcus saprophyticus* *Staphylococcus sciuri*

3.3.1.2 Smoked salmon factory

The production of smoked salmon is a high risk process as the product is not cooked prior to consumption. The movement of the fish through the processes involved are shown in Figure 3.1. On arrival from the wholesaler, frozen fish were left to thaw, but there was no control over temperatures reached, therefore the fish at the surface were at temperatures conducive to bacterial growth. The viable counts show that 4.70×10^2 cell cm^{-2} were present on the thaw tank wall (Table 3.3).

After deheading the fish were filleted. The low count on the fillet machine blades, compared to the rubber rollers in the fillet machine, highlights the differences in cleanability of the two surface types partially due to the differences in material types and partially due to accessibility. The rollers touched every fish that passed through the machine and therefore contaminated them.

The fillets entered one side of the smokers, and after smoking exited through the other side so that there is no movement of untreated fish to the slicing and packing area. Both the slicing table and knife have counts of approximately 10^2 cells cm^{-2}. The water standing on the lateral slicer gave high counts of 1.50×10^4 cells cm^{-2}. It is worthy of note that problems with condensation and poor drainage have been implicated in outbreaks of *Listeria* food poisoning.

The bacteria identified were predominantly *Staphylococcus* species with the ubiquitous Pseudomonads also present, as well as *Acetobacter calcoaceticus* and *Serratia* sp (Table 3.5). However, the data showed similar organism combinations throughout the factory, *Staphylococcus* species being more common in the manual stages of the system; slicing, de-boning and weighing.

Figure 3.1

Flow diagram of the processes involved in smoked salmon production

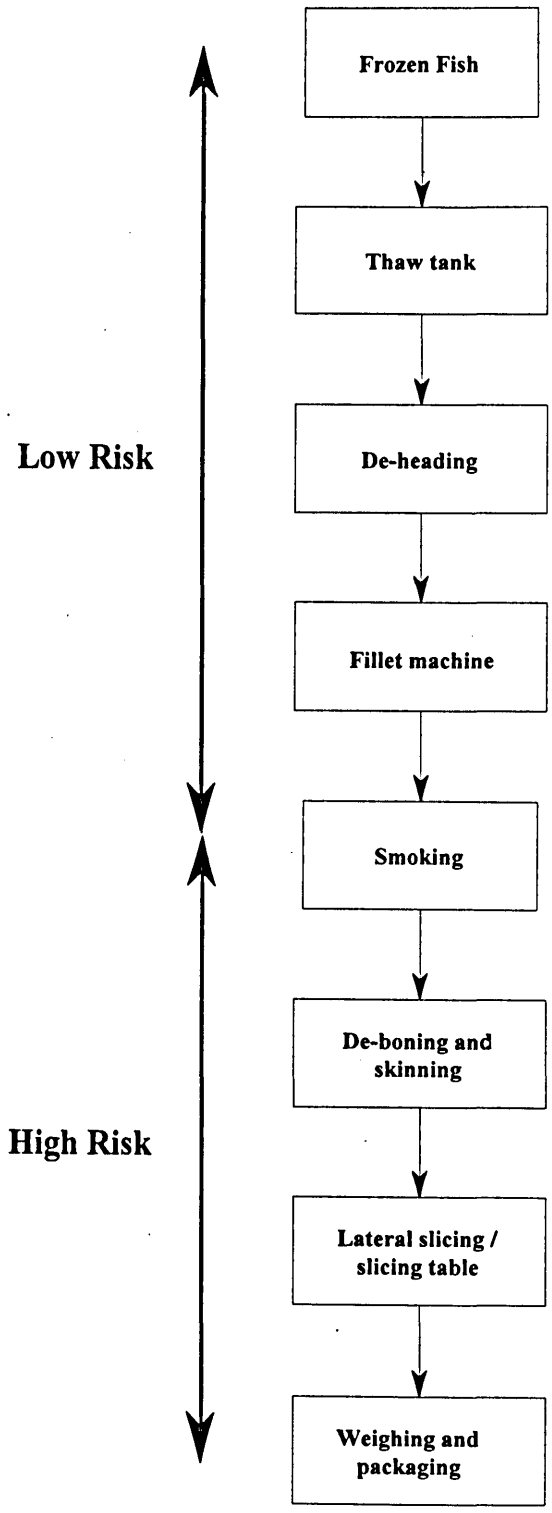


Table 3.3

Viable counts per cm^2 on the surfaces sampled in the smoked salmon factory

Sample site	Total	Coliform	Fastidious	Staph.
Hand wash sink	$5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Fish slab	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
SS table	$<5.00x10^0$	$<5.00x10^0$	$5.00x10^0$	$<5.00x10^0$
SS table	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Equipment sink	$1.00x10^1$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Hand wash sink	$2.50x10^2$	$<5.00x10^0$	$5.00x10^0$	$<5.00x10^0$
SS boning table	$6.00x10^1$	$<5.00x10^0$	$1.00x10^2$	$<5.00x10^0$
Teeth on de-skinner	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Conveyer belt on slicer	$3.00x10^1$	$5.00x10^0$	$5.00x10^0$	$<5.00x10^0$
Trolley	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Fish tray	$5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Blades in slicer	$1.00x10^1$	$1.20x10^2$	$<5.00x10^0$	$<5.00x10^0$
Water on slicer	$1.50x10^4$	$1.20x10^2$	$<5.00x10^0$	$<5.00x10^0$
SS slicing table	$1.20x10^2$	$<5.00x10^0$	$2.60x10^2$	<5.00x100
Slicing knife	$1.30x10^2$	$3.00x10^1$	$5.00x10^1$	$6.00x10^1$
Weighing table	$1.00x10^1$	$<5.00x10^0$	$1.00x10^1$	$2.50x10^1$
Balance	$<5.00x10^0$	$<5.00x10^0$	$2.50x10^1$	$1.00x10^1$
Thaw tank	$4.70x10^2$	$4.25x10^2$	$4.85x10^2$	$1.38x10^2$
Fillet machine blade	$5.00x10^0$	$5.00x10^0$	$1.00x10^1$	$<5.00x10^0$
Rollers in fillet machine	$1.50x10^4$	$1.50x10^4$	$1.50x10^4$	$1.50x10^4$
SS deheading table	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
Balance for fresh fish	$<5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$	$5.00x10^0$
Cold store wall	$<5.00x10^0$	$5.00x10^0$	$<5.00x10^0$	$<5.00x10^0$
SS smoker door	$<5.00x10^0$	$<5.00x10^0$	$5.00x10^0$	$<5.00x10^0$

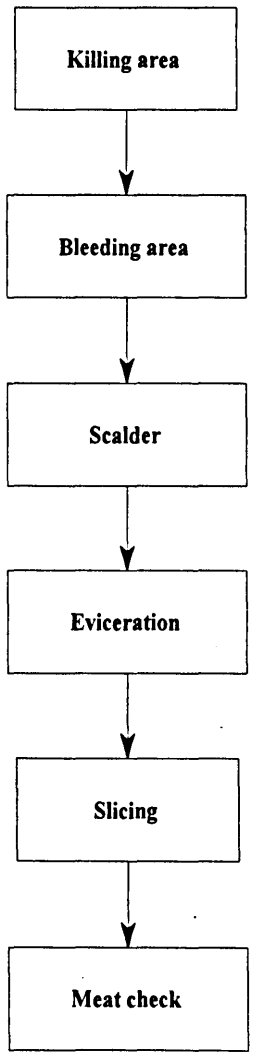
Limit of detection $<5.00x10^0$

3.3.1.3 Pig abattoir

The movement of the carcasses through the stages involved are shown in Figure 3.2. The data from the abattoir showed that generally there were higher numbers of bacteria present than at either of the other sample sites. Both the tile and the stainless steel surface in the bleeding area had between 10^3-10^4 cells cm^{-2}, since it is a highly nutritious environment and the environmental temperature may be slightly elevated due the presence of the fresh carcasses.

Figure 3.2

Flow diagram of the processes involved in the abattoir



After bleeding the carcasses entered the scalder to remove the bristles, the temperature of the water in the scalder should be in excess of 82°C and therefore present little microbiological hazard, however in reality the temperature of the scalder may in fact be any temperature from ambient upwards. The plastic hatch at the exit of the scalder had relatively high bacterial counts, and as this flap touched every carcass it therefore contaminated the surface of every carcass it touched.

The data showed that the cleaning procedure produces a log reduction in the number of bacteria present on the saw blade.

After grading, the carcasses entered the chiller. The swab samples from the chiller wall and its stainless steel skirting showed the differences in the cleanability of the two surface types.

As with the salmon factory, similar organisms were found throughout the abattoir. They were predominantly *Staphylococcus* species of animal origin, but also present were Pseudomonads and *E.coli* at one sample site probably due to poor removal of the intestines of the pigs leading to contamination of the environment.

Table 3.4

Viable counts per cm^2 on the surfaces in the pig abattoir.

Site description	Total	Coliform	Fastidious	Staph.
Bleeding area wall tile	$4.78x10^3$	$5.63x10^2$	$3.44x10^3$	$5.50x10^2$
Bleeding area - SS surface	$2.26x10^4$	$3.26x10^3$	$1.24x10^4$	$2.94x10^3$
Equipment sink	$<5.00x10^0$	1.25x100	$1.25x10^0$	$2.50x10^0$
Throat knife	$1.44x10^2$	$1.13x10^0$	$7.00x10^1$	$6.25x10^0$
Evisceration knife	$1.60x10^2$	$1.25x10^0$	$3.58x10^2$	$2.50x10^0$
Saw blade - pre cleaning	$2.50x10^1$	$7.50x10^0$	$3.00x10^1$	$5.00x10^0$
Saw blade - post cleaning	$2.50x10^0$	$1.25x10^0$	$<5.00x10^0$	$<5.00x10^0$
Chiller wall - SS surface	$3.13x10^1$	$1.25x10^0$	$3.25x10^1$	$3.75x10^0$
Chiller wall - concrete	$3.63x10^2$	$2.93x10^2$	$4.50x10^2$	$6.25x10^0$
Plastic flap in scalder	$5.00x10^3$	$2.18x10^3$	$1.16x10^4$	$2.88x10^3$
Hanging rack -post cleaning	$2.90x10^2$	$1.14x10^2$	$1.78x10^2$	$5.50x10^1$

Limit of detection $<5.00x10^0$

Table 3.5

Bacteria found on surfaces in the salmon factory and abattoir

Factory environment	Bacteria identified
Smoked salmon factory	*Staphylococcus hominis*
	Staphylococcus cohnii
	Staphylococcus sciuri
	Staphylococcus xylosus
	Staphylococcus saphrophyticus
	Serratia fonticola
	Serratia liquefaciens
	Pseudomonas sp.
	Pseudomonas fragi
	Acetobacter calcoaceticus var lwoffi
Pig abattoir	*Staphylococcus sciuri*
	Staphylococcus epidermidis
	Staphylococcus cohnii
	Staphylococcus hominis 1
	Staphylococcus hominis 2
	Pseudomonas sp.
	Escherichia coli
	Serratia sp.

3.3.1.4 Conclusions from the factory surveys

Characterization of the bacteria present on surfaces within the food industry showed that the biofilm communities from the environments sampled ranged from relatively homogeneous to very heterogeneous populations. Analysis of the community composition used standard microbiological techniques and therefore was restricted to organisms that grew readily in the laboratory. Work characterizing the 16S ribosomal RNA sequences from naturally occurring biomasses have shown that the organisms revealed are more diverse than originally thought due to the presence on unculturable organisms (Olsen, 1990). It was concluded that the culturable bacteria detected in this study were probably the more important components of the consortia in terms of growth and development of biofilms. However, the quiescent cells, by their nature may be more resistant to biocides and remain on the surfaces.

The level of surface contamination varied with product-type and environment, from no detectable organisms to 2.26×10^4 cells cm^{-2}. These levels are comparable to those found by other workers in similar environments. Lewis and Gilmour (1987) found similar levels in a dairy environment; 8.13×10^3 mesophiles per cm^2 on stainless steel and 3.47×10^4 mesophiles per cm^2 on rubber surfaces. However, swabbing is a relatively inefficient method of removing bacteria from surfaces, and certain types remain attached. Consequently the counts and identifications are only of species removed from the surfaces by swabbing. Holah *et al.* (1989) determined the number of organisms on surfaces in a wide variety of product-type environments using direct epifluorescent microscopy (DEM). Stainless steel coupons were attached to the equipment for various time periods, after which the coupons were removed and stained with the fluorescent dye acridine orange for examination using an epifluorescent microscope linked to an image analysis system. They found approximately 10^4 cells cm^{-2} on surfaces in a fish filleting operation which is a similar to the maximum levels found in the smoked salmon factory by swabbing. The DEM count was a total count of the bacteria *in situ* and compares favourably with the results in this study obtained by swabbing.

In conclusion, although the viable count may be an underestimate of the surface population due to the inaccuracies of swabbing and the presence of unculturable cells, the data showed the differences in the relative populations in the varied environments.

A relatively simple mixture of three species was chosen for attachment studies and to model biofilm development in the laboratory The bacteria chosen were *Serratia liquefaciens, Pseudomonas fragi* and *Staphylococcus cohnii* which were all found on the stainless steel surface in the smoked salmon factory. This particular combination was chosen since it comprised both Gram negative and positive organisms of differing morphology - rods and cocci - and represented three different genera.

3.3.2 Characterization of selected organisms

The cell size, morphology, Gram reactions and motility were determined for the isolated component species of the chosen biofilm - *S.liquefaciens, P.fragi* and *S.cohnii* (Table 3.6). The identification profiles of the organisms obtained from API Identification test strips are shown in Tables 3.7 and 3.8. The identification percentage is an indication of the certainty of the result, and therefore the identification of *S.cohnii* had a high degree of certainty. Although the percentages for *S.liquefaciens* and *P.fragi* were lower, the percentages to genus level (i.e. *Serratia* and *Pseudomonas*) were in excess of 95%.

Serratia and Pseudomonas species are primarily found in the natural environment in soil and water, whilst *Staphlyococcus* species form part of the normal flora of the human skin. Often *Serratia* species produce characteristic pigments e.g. prodigiosin by *Serratia marcescens,* however, *S. liquefaciens* is a non-pigmented organism.

Figure 3.3 shows the optimum growth temperatures of the organisms. *S.cohnii* has an optimum growth temperature around 25°C, *S.liquefaciens* 30°C and *P.fragi* 37°C.

Figure 3.4 shows the pH optima of the isolates. These were determined by adding a standard inoculum to LB broth adjusted to pHs between 5 and 10. The optical densities were read after 5 hours as the cell density was not sufficient by this stage to have changed the pH of the medium significantly but gave an indication of the optima pH ranges for the three organisms (Table 3.9). *S.liquefaciens* had an optimum between 7.5-8 although this organism appeared to by relatively unaffected by pH as determined by this simple method. The optimum pH for growth of *P.fragi* was between 6.5-7 and between 7.5-8 for *S.cohnii.*

A temperature of 25°C was chosen for subsequent attachment studies and biofilm modelling as this temperature is close to that found in a factory environment running at ambient temperature. Generally experiments were conducted at a pH of 7.4 as this was close to the optimum pH for all three organisms.

Table 3.6

Characteristics of *S.liquefaciens*, *P.fragi* and *S.cohnii.*

Characteristic	***S.liquefaciens***	***P.fragi***	***S.cohnii***
Cell morphology	short rod	rod	cocci
Cell size	0.5 x 1.2 μm	0.75 x 2.0 μm	0.75 μm
Gram reaction	Negative	Negative	Positive
Motility	Peritrichous flagella	Polar flagellum	
Catalase reaction			Positive
Oxidase reaction	Negative	Positive	

Table 3.7

API 20E identification profiles for *S.liquefaciens* and *P.fragi*

Biochemical test	***S.liquefaciens***	***P.fragi***
Beta-galactosidase	+	-
Arginine dihydrolase	-	+
Lysine decarboxylase	+	-
Ornithine decarboxylase	+	-
Citrate utilization	+	+
H_2S production	-	-
Urease	-	-
Tryptophan desaminase	-	-
Indole production	-	-
Acetoin production	+	-
Gelatinase	+	+
Glucose utilization	+	+
Mannitol utilization	+	-
Inositol utilization	+	-
Sorbitol utilization	+	-
Rhamnose utilization	-	-
Sucrose utilization	+	-
Melibose utilization	+	-
Amygadalin utilization	+	-
Arabinose utilization	+	+
Cytochrome oxidase	-	+
% Identification	79.3	75.4

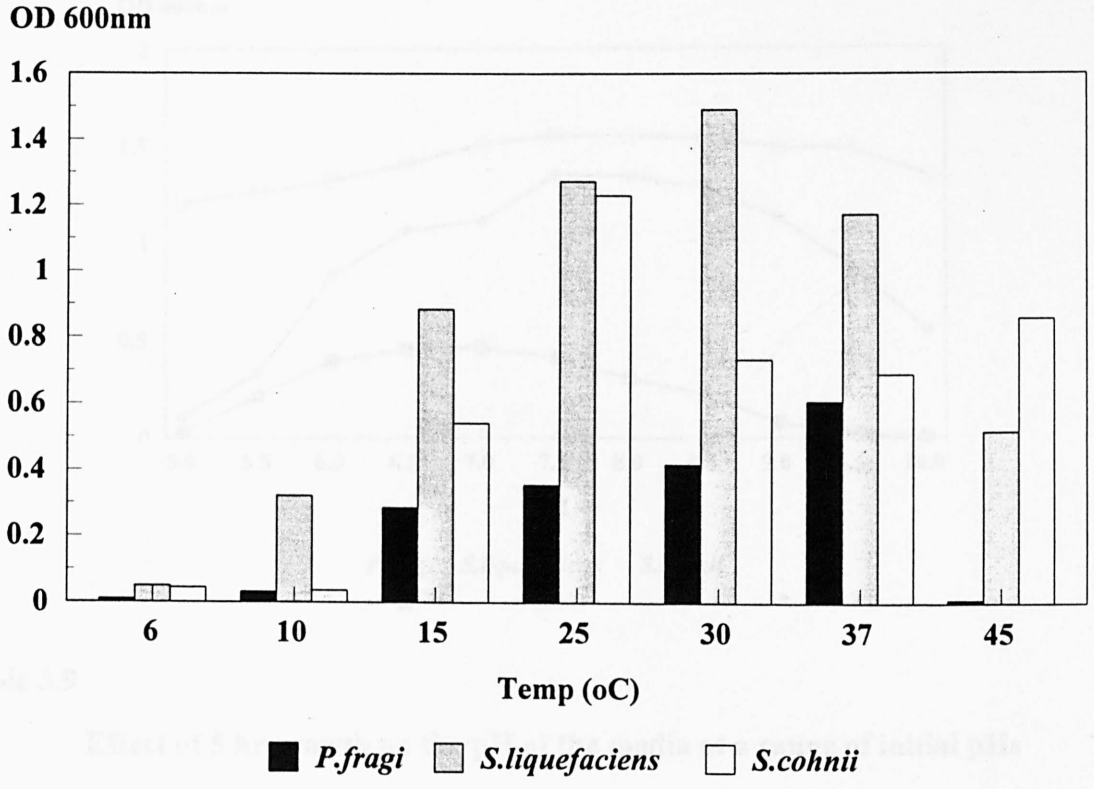
Table 3.8

API STAPH identification profile for *S.cohnii*

Biochemical test	***S.cohnii***
Glucose utilization	+
Fructose utilization	+
Mannose utilization	+
Maltose utilization	+
Lactose utilization	-
Trehalose utilization	+
Mannitol utilization	-
Xylitol utilization	-
Melibiose utilization	-
Nitrate reduction to nitrite	-
Alkaline phosphatase	+
Acetoin production	+
Raffinose utilization	-
Xylose utilization	-
α-methyl-D-glucoside utilization	-
N-acetyl-glucosamine utilization	-
Arginine dehydrogenase	-
Urease	-
Lystostaphin resistance	-
% Identification	98.5

Figure 3.3

Comparison of the optimum growth temperatures of the three selected strains



The bacteria were grown in LB broth at the specified temperatures for 16 hr and the optical densities at 600nm recorded. The media was equilibrated to the appropriate temperatures before inoculation.

Figure 3.4

Comparison of the optimum growth pHs of the selected strains

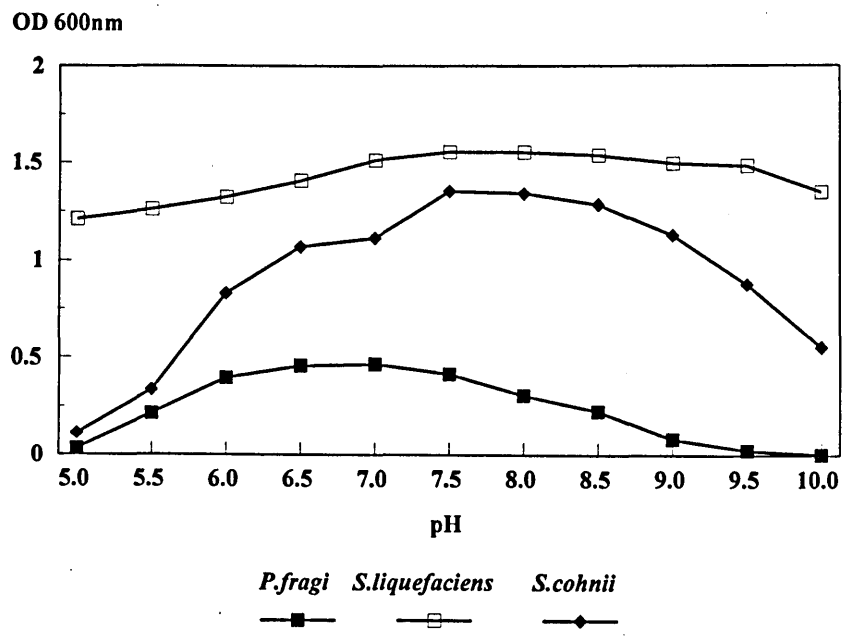


Table 3.9

Effect of 5 hr growth on the pH of the media at a range of initial pHs

Initial pH of media	*P.fragi*	*S.liquefaciens*	*S.cohnii*
5.0	5.0	5.0	5.0
5.5	5.5	5.5	5.5
6.0	5.8	5.9	6.0
6.5	6.3	6.4	6.2
7.0	6.9	6.7	6.9
7.5	7.4	7.2	7.3
8.0	7.8	7.9	7.7
8.5	8.4	8.5	8.3
9.0	9.0	9.0	8.8
9.5	9.5	9.5	9.4
10.0	10.0	10.0	10.0

CHAPTER FOUR

4. ATTACHMENT STUDIES AND BIOFILM DEVELOPMENT

4.1 Introduction

Food processing equipment and its environment have a variety of material types used in their construction including stainless steel, nylon, polypropylene, and polyvinyl chloride. Bacteria are able to attach to and grow on a wide range of surface types. Holah and Thorpe (1990) observed total surface coverage after four hours on stainless steel, polycarbonate, high density nylon, enamelled steel, aluminium and mineral resin.

The most commonly used material is stainless steel due to its corrosion and abrasion resistance, malleability and cleanability (Timperley *et al.*, 1992). There are several grades of stainless steel but type 2B cold rolled steel is used for general applications within the food industry. The method used to finish the steel dictates its roughness and varies from 0.2 μm Ra (where Ra is roughness average) for electropolished stainless steel to 1.15 μm Ra for bead blasted steel. Type 2B stainless steel has a surface roughness value of 0.2 μm Ra.

All plants manufacturing wet products are liable to host biofilms. All unsterilized equipment harbours bacteria which between two cleaning and disinfection operations have time to develop into biofilm consortia.

The conditions in the food processing environment, in terms of type of surface, type of product and temperature, will exert selection pressures on the organisms present and affect the biofilm that develops. Lewis and Gilmour (1987) showed that among the bacteria present in raw milk, it was mainly the Gram negative bacteria that attached to the processing equipment, although the milk microbial flora was predominantly Gram positive bacteria. This data demonstrates the differences in attachment potentials between species.

Bacteria are far more resistant to anti-microbial agents when part of a biofilm or attached to a surface than those dispersed in a liquid medium. Holah *et al.* (1990) showed that the concentration of disinfectant required to achieve the same effect as that observed for suspended bacteria was between ten and a hundred times greater. Anwar *et al.* (1992) reported that the resistance of the biofilm to biocides increased with the age of the biofilm.

The increased resistance of biofilm bacteria to biocides may be due to protection of the cells by the extracellular polymeric matrix. Oxidizing biocides, such as chlorine releasing compounds, react with organic matter including EPS, and therefore the concentration of oxidising agent available to affect the bacterial cell is reduced. Bolton *et al.* (1988) observed that the resistance of *Staphylococcus aureus* in suspension increased when the organism had been cultured in a medium that favoured the synthesis of extracellular polymer.

Alternatively, the process of adhesion may involve physiological changes which enhance the resistance of the cell to anti microbial agents (Nichols, 1989).

The presence of polymer producing organisms on surfaces in the processing environment poses significant problems for the cleaning and disinfection regimes. As organisms attached to surfaces are more resistant to biocides, the bacteria may remain on the surface in the food processing environment as a source of spoilage and or pathogenic organisms. Understanding the attachment of bacteria to stainless steel is the first step in being able to develop methodologies to reduce or eliminate this problem.

This chapter examines the attachment of *S.liquefaciens, S.cohnii* and *P.fragi* to stainless steel and describes a system designed to model biofilm development in the laboratory using a mixed culture of these three organisms.

4.2 Aims

The objectives of this section were two fold. The first was to examine the effects of a range of environmental conditions such as temperature, pH and anerobiosis on the attachment of the three species. Secondly, the study modelled mixed culture biofilm development and examined the involvement of the individual species in that consortium. The primary objective was to evaluate the effects of growth rate and nutrient limitation on mixed culture biofilm development on stainless steel.

4.3 Static attachment assays.

4.3.1 Effect of pH on attachment

The effect of pH on attachment was examined in two ways :-

(i) Cells were harvested and resuspended in buffer at different pHs

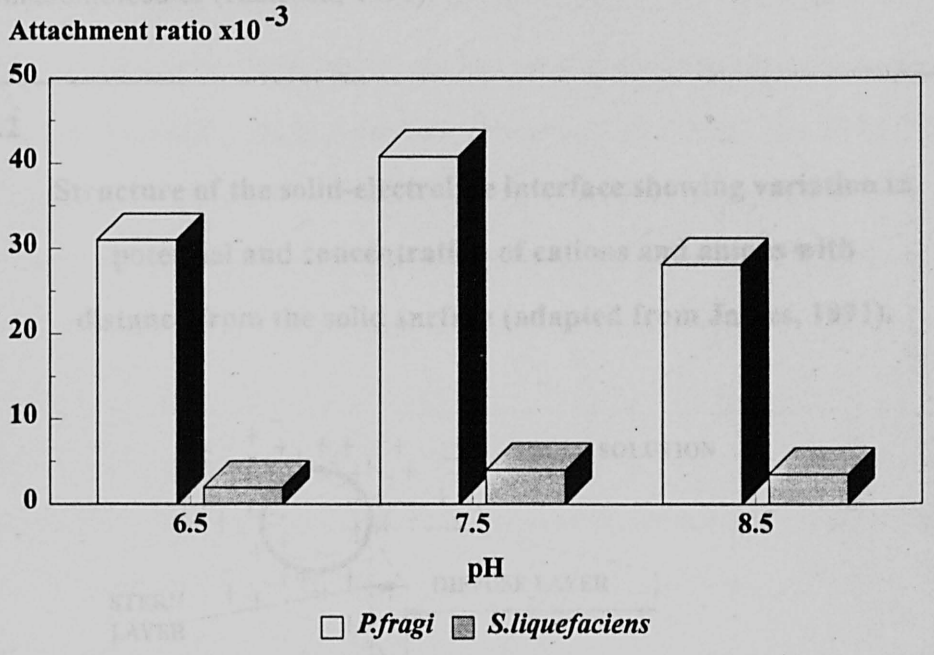
(ii) Cells were grown at different pHs.

4.3.1.1 Effect of pH of the buffer on attachment

Figure 4.1 shows that attachment was maximal at pH 7.5 for *P.fragi* and *S.liquefaciens*. However, it should be noted that only a narrow range of pHs were tested.

Figure 4.1

Effect of buffer pH on attachment ratios

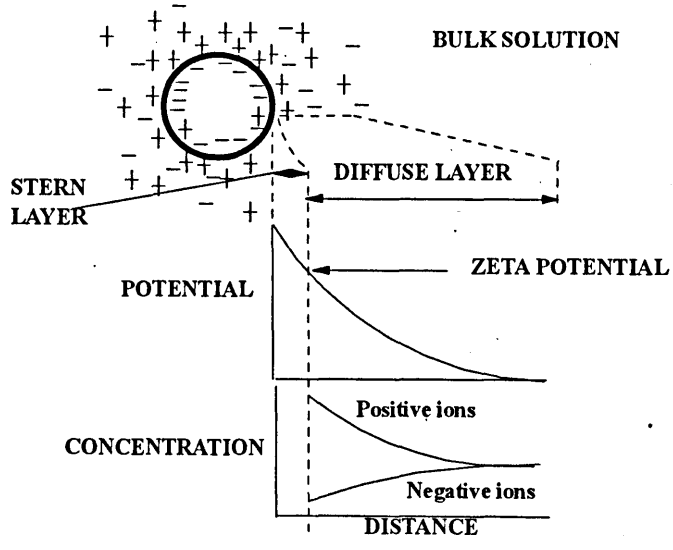


Cells grown in carbon and nitrogen excess media at pH 7.5 at 25°C were resuspended in buffer at different pHs to a standard cell density and the number of cells attached to triplicate stainless steel discs was determined after 1 hr by direct epifluorescent microscopy (twenty fields of view counted on each disc - section 2.13). Attachment ratios were calculated by dividing the surface concentration by that in the liquid phase.

The majority of solid bodies acquire an electrical charge when immersed in an aqueous medium, due to ion adsorption or ionization of the surface groups which is dependant upon the pH of the medium. A charged surface immersed in an aqueous medium will promote a redistribution of ions - co-ions will be repelled from the surface and counter-ions attracted (Figure 4.2). This results in a distribution of ions in the aqueous phase creating a diffuse layer. The electrostatic potential decreases from the surface through this diffuse layer attaining a null value in the bulk phase. In the physiological pH range, bacteria possess a net negative charge. However, the isoelectric point of bacteria is towards acidity, therefore at low pH values, bacteria may possess a net positive charge. Charge reversal at low pH may also occur due to the presence of basic amino groups. Negative charges occur due to the presence of phosphate, carboxylate and less commonly sulphate groups in the cell wall and capsular macromolecules (Hancock, 1991).

Figure 4.2

Structure of the solid-electrolyte interface showing variation in potential and concentration of cations and anions with distance from the solid surface (adapted from James, 1991).



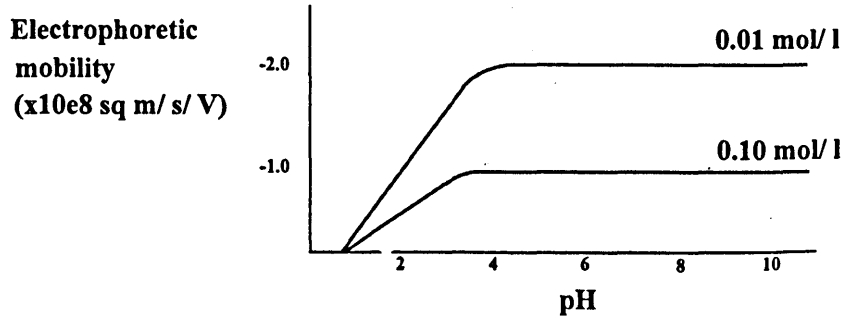
Electrophoretic mobility, which is a measure of the net surface charge, is dependant on pH with the pH-mobility curve varying between organisms. The curve for *Klebsiella aerogenes* is relatively simple as the charge is due to one charged species only, whereas the situation is more complicated for organisms with multiple iogenic groups (James, 1991).

As a consequence of the above, bacterial attachment may be expected to be higher at lower pH values as a reduction in the net negative charge of the cell would decrease the electrostatic repulsion between the cell and the negatively charged stainless steel surface. However these data show that levels of attachment were significantly higher at pH 7.5, particularly for *P.fragi.* This would suggest that modification of the electrostatic double layer is not responsible.

The optimum growth pH for *P.fragi* is approximately 7 and for *S.liquefaciens* 7.5. Stanley (1983) suggested that active transport of cations to the cell surface may create less negatively charged outer cells and this would occur at the highest rate at the optimum growth pH. According to Stanley (1983) maximal attachment of *P.fragi* and *S.liquefaciens* would therefore occur at pH 7 and 7.5 respectively. The attachment period used was 1 hour and therefore, there was limited time for pH to have an effect. Perhaps it is not unexpected that the optimum attachment pH was the same as that at which the cells were grown.

Figure 4.3

pH mobility curves for *Klebsiella aerogenes* in acetate-barbiturate buffer at different ionic strengths (adapted from James, 1991)



4.3.1.2 Effect of growth pH on attachment ability

This data was obtained for cells grown at the pH values under test, whereas in Figure 4.1 cells were grown in media at pH 7.5, were washed and resuspended in buffer at different pH values.

Figure 4.4 shows that the growth pH that produced the maximum level of attachment was 6.5 for both *P.fragi and S.liquefaciens*. These data are different to the attachment data in buffers of different pH which showed that the greatest levels of attachment occurred, in buffer, at pH 7.5.

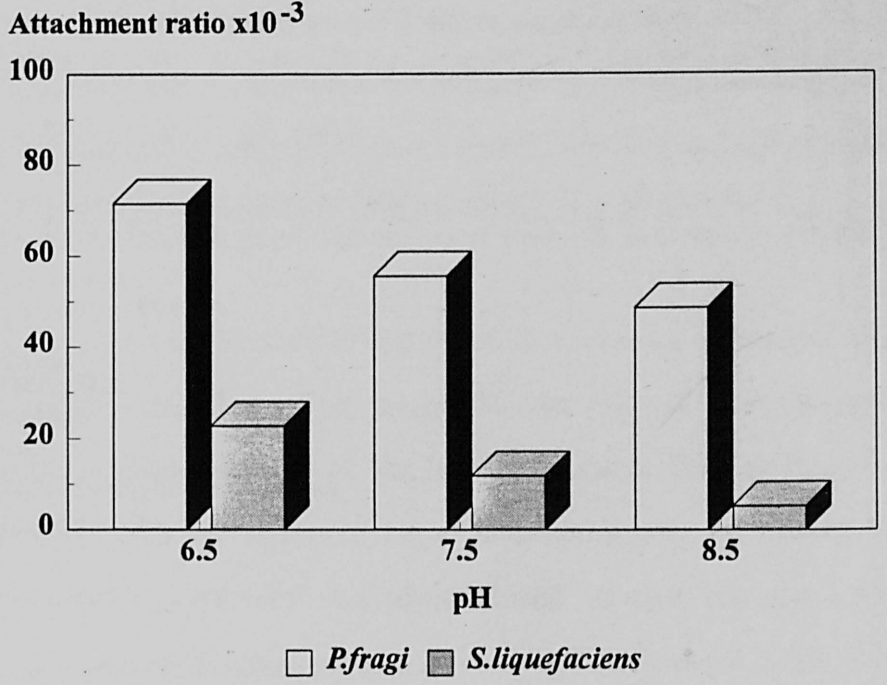
As already described bacterial cells are negatively charged at neutral pH, but become less negatively charged at low pHs. The reduction in pH causes a redistribution in the ions in the diffuse layer around the cell and may result in reduced electrostatic repulsion between the cell and the surface, and therefore favour attachment.

These data suggested that electrostatic interaction was of importance since maximum attachment was found at the lowest pH.

Modification of the electrostatic double diffuse layer may not be solely responsible, as growth pH may have a significant effect on expression of cell surface characteristics. Ellwood and Tempest (1972) describe the effects of growth pH on cell wall content and composition of *Bacillus subtilis*. In this work the culture medium composition affected the extent of substitution of Gram positive cell wall components with alanyl ester groups and hence the net charge of both cell wall teichoic acids and lipoteichoic acids. pH has also been shown to influence the balance between teichoic and teichuronic acids in the cell wall, flagellum formation in *Proteus* and encapsulation of *Streptococcus pneumoniae* (Ellwood and Tempest, 1972 ; Hancock and Poxton, 1988). Also, Herald and Zottola (1988) found that the production of fibrillar material between cells and the surface was affected by pH. However, the attachment periods were 18-36 hr and therefore production of extracellular material could have played a significant role. In this study the attachment period was 1 hr, however, the production of extracellular material, which was not lost by washing, could have played a role and may have been affected by the growth pH in terms of both nature and quantity.

Figure 4.4

Effect of growth pH on attachment ratios



Cells were grown in carbon and nitrogen excess media at 25°C at the specified pHs, harvested and resuspended in buffers to a standard cell density, and the numbers of attached organisms after a one hour attachment period were enumerated by direct epifluorescent microscopy (section 2.14). Triplicate stainless discs were used and 20 fields of view per disc were counted.

Various workers have reported variation in adherence of micro-organisms to inert surfaces depending upon the pH of the medium. Wood (1980) found that Gram positive cells had increased adsorption to cation exchange resin beads at pH 4.5-5.4 as compared to pH 5.5-7.5. It was also found that Gram negative organisms adhered better at the higher pH range. Stanley (1983) found that *P.aeruginosa* cells attached to stainless steel in greatest numbers at pH 7-8 and concluded this range was where metabolic activity and therefore attachment was greatest. This compares very well with the attachment data of *P.fragi* to stainless steel observed in this study.

McEldowney and Fletcher (1988a) found that maximal attachment of *Pseudomonas fluorescens* occurred at pH 7. They suggested that pH may have affected adhesion by influencing the physico-chemistry of the interaction rather than by directly affecting the physiology of the cells i.e. effects of pH on attachment may be through influencing the viscosity of various polymers. Sutherland (1983) showed that the exopolysaccharide produced by a fresh water bacterium had a maximum viscosity at pH 7, and decreased at pH 3 and 11. Boyle and Reade (1983) demonstrated that the viscosity of exopolysaccharide produced by a marine isolate decreased as the pH increased. At lower pHs, therefore the polymers would be more viscous and possibly less easily removed by washing. In this study washed cells were resuspended in buffer for the attachment assay, if the cells grown at lower pH had a more viscous polysaccharide then more would remain to interact with the surface in the attachment assay.

Lewis *et al.* (1989) evaluated the effect of pH on the detachment of cells from stainless steel. Increasing the pH was found to increase numbers of detached bacteria. The data indicated that the attachment and detachment of *Acinetobacter* sp in these experiments involved an acidic polysaccharide. From this, it was postulated that changes in pH would influence the number of charges on the molecules of extracellular polysaccharide leading to conformational changes, and consequently affecting attachment.

The effect of pH on hydrophobicity, cell surface charge, polysaccharide production and outer membrane proteins are examined in subsequent chapters.

4.3.2 Effect of temperature on attachment

The effect of temperature on attachment was examined in two ways :-

(i) Cells grown at 25 ^{o}C were washed and resuspended in buffer at different temperatures and the hour attachment period was at the temperature under test.

(ii) Cells were grown at a range of temperatures for the attachment assay.

4.3.2.1 Effect of assay incubation temperature on attachment

Figure 4.5 shows that the temperature of incubation of the attachment assay does not have a significant effect on the attachment of *P.fragi* and *S.liquefaciens* to stainless steel. It may simply be that the incubation time of 1 hour was too short for the temperatures tested to have a noticeable effect on the attachment. However, the short time in buffer means that there was limited change in cell concentration, and therefore differences between the numbers of cells on the surfaces were due to the direct effects of the parameter under test i.e. temperature or pH and not to an indirect effect resulting from an increase in the cell concentration. Attachment ratios were calculated by dividing the cell concentration on the surface (cells cm^{-2}) by the concentration of cells in the liquid phase (cells ml^{-1}) as this allows for slight differences in liquid phase concentration.

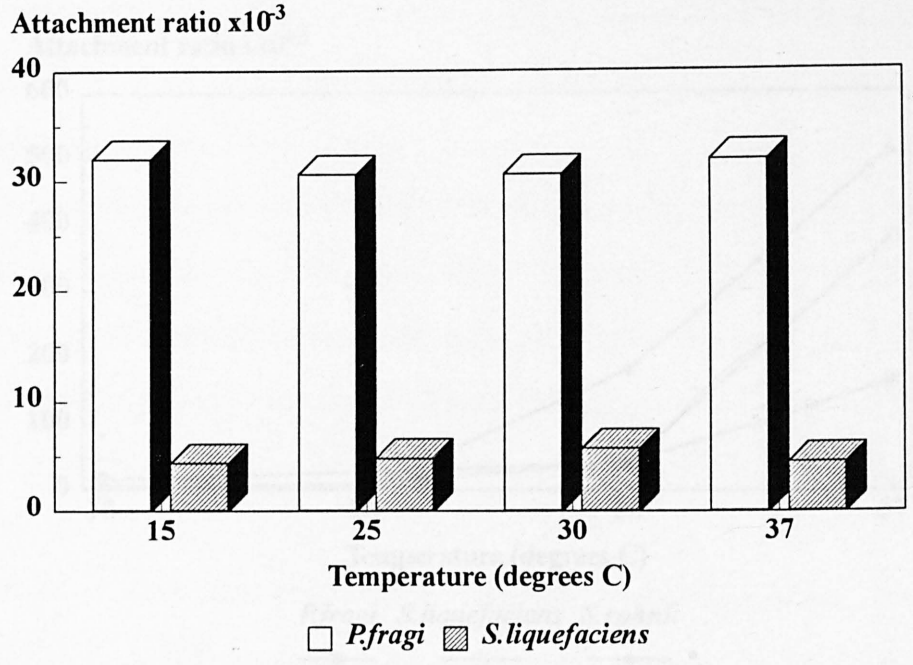
As there is no difference between attachment at the different temperatures, initial bacterial attachment cannot be determined by physicochemical adsorption since chemisorption is higher at higher temperatures.

4.3.2.2 Effect of growth temperature on attachment ability

Figure 4.6 shows that increasing the growth temperature increased the number of attached cells of *P.fragi, S.cohnii* and *S.liquefaciens.* The attachment assay was for one hour in buffer so that the cell concentration remained relatively constant for the assay, and any slight differences between inocula were accounted for by calculating attachment ratios (cells cm^{-2} divided by cells ml^{-1}).

Figure 4.5

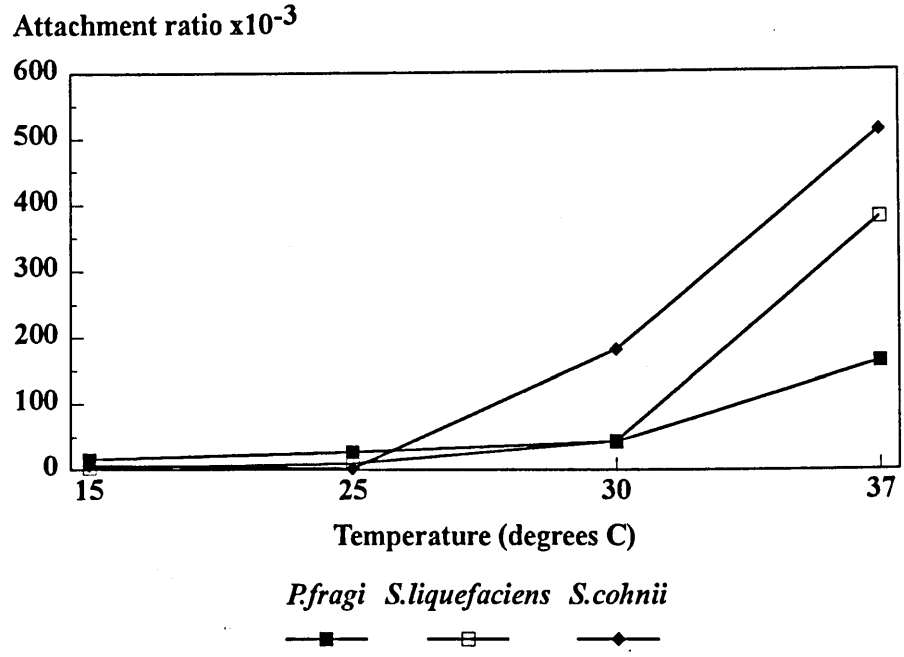
Effect of assay incubation temperature on attachment



Cells were grown at 25°C in carbon and nitrogen excess media at pH 7.5, harvested and resuspended in buffer at the specified temperatures. The number of attached cells was determined after one hour by direct epifluoresecent microscopy (section 2.14) and attachment ratios calculated. Triplicate stainless steel discs were used and 20 field/disc were counted.

Figure 4.6

Effect of growth temperature on attachment



Cells were grown at the specified temperatures in carbon and nitrogen limited media, harvested and resuspended in buffer. The number cells attached to the stainless steel was determined by direct epifluorescent microscopy and attachment ratios calculated. Triplicate stainless steel discs were used and 20 fields/disc counted.

The attachment ratios at 25°C are directly comparable to those in Figure 4.5 which evaluated the effect of incubation temperature on attachment. The cells for the latter experiment had been grown at 25°C before washing and resuspending in buffer at the specified temperatures. It can be seen that similar values were obtained; mean attachment ratios (n=3) for *P. fragi* in the two experiments were 27.3 and 35.6. This reinforces the reproducibility of the assay.

Maximum attachment was found at 37°C, although the optimum growth temperatures of the organisms are 25°C, 30°C and 37°C. It is possible that the different growth temperatures may have produced a change in the cell surface characteristics thus altering the attachment abilities of the organisms. These characteristics will be inclusive of outer membrane proteins, extracellular polysaccharides (type, quantity and viscosity) flagella and fimbriae.

Various workers have examined bacterial attachment at a range of temperatures. Herald and Zottola (1988) observed morphological differences in attached cells at different temperatures e.g. *Listeria monocytogenes* attached to stainless steel at 35°C was present as single cells or clumps with many cells possessing flagella, however at 21°C single cells were present with multiple flagella and the few cells present at 10°C did not have flagella. Flagellated cells may attach more readily as these structures may aid contact with the surface and overcome the electrostatic repulsion forces (Fletcher, 1979). However, McSweegan and Walker (1986) did not detect a difference between flagellated and non flagellated cells. Phase contrast examination of cells grown at the different temperatures did not show marked differences in the level of motility (*S.cohnii* is a non motile organism) therefore differences in flagella expression does not explain the observed differences in attachment ability at different temperatures. *S.cohnii* does not produce flagella but does exhibit the same increase in attachment ratios with increasing temperature.

McEldowney and Fletcher (1988a) found that maximum attachment of *Pseudomonas fluorescens* occured at 25°C, this temperature is also the optimum growth temperature for this organism. The data presented in this thesis for *P. fragi* showed a similar trend in that the maximum level of attachment occurred at the optimum growth temperature.

Temperature, as well as pH, affects the viscosity of polymers and, therefore, adhesion. The efficiency of some attachment polymers decreased with increased temperature due to a decline in their viscosity (McEldowney and Fletcher, 1988a). McEldowney and Fletcher (1988a) suggested that the reduction in the permanent adhesion of a *Flexibacter* species with increasing temperature could be explained by a decrease in adhesive viscosity. Fletcher (1977) also suggested that the attachment efficiency could be reduced at 3°C because of an increase in the viscosity of the medium or of the bacterial surface polymer.

Higher temperatures favour chemisorption and certain types of physical adsorption. If bacterial adhesion is initially by physico chemical adsorption, then decreased adhesion would be observed at lower temperatures. This theory ties in with the observed data as all three organisms showed increases in attachment ratios with increasing temperature. However, if this is the case then the experiments looking at attachment of cells in buffer at different temperatures should have shown the same trend. There are possibly two explanations for this discrepancy. Firstly, the cells were not sufficiently equilibrated to the buffer temperatures before the attachment assay. Secondly chemisorption may play a role but growth at the different temperature results in different cell surface characteristics - 37°C producing cells with the highest attachment potential. However, it is unlikely that the same temperature would produce the most adhesive cells in all three species. If temperature influences the adhesion by affecting the physiology of the micro-organisms, then the attachment ability of the organism at different temperatures would be expected to mirror the temperature growth profile of the organism.

The effect of temperature on hydrophobicity, cell surface charge, polysaccharide production and outer membrane expression are examined in subsequent chapters.

4.3.3 Effect of nutrient limitation and growth phase on the attachment of *S.liquefaciens*

Figures 4.7 A-D show the attachment of *S.liquefaciens* through the growth cycle and in different media. The numbers of cells attached to the stainless steel was highest for cells grown in the complex medium (LB) during the late exponential phase. The attachment ratio

was significantly lower in stationary phase LB grown cells. The other media used were carbon limited, nitrogen limited and carbon and nitrogen excess. (Obviously in batch culture these designations do not hold strictly true.) Carbon limited media contained low levels of carbon and high levels of nitrogen, whilst for the nitrogen limited media the reverse was true.

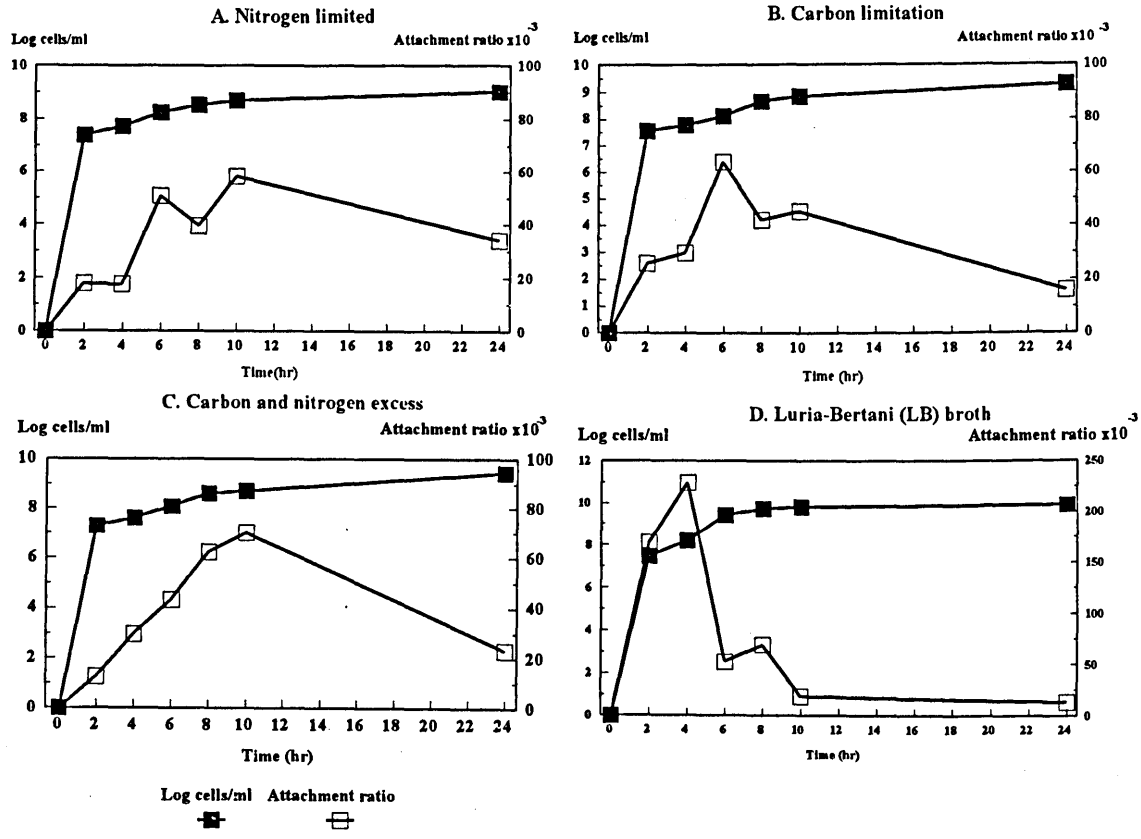
The complex medium may have physiological effects on the liquid surface tension because of the interaction between water molecules and solutes, but the macromolecular components in the complex medium will also form a conditioning layer on the substratum, thus altering the surface characteristics. In the attachment assay, cells were harvested by centrifugation, washed twice in buffer and resuspended in buffer for the attachment assay. Consequently the majority of the medium components will have been removed by the washing procedure, although some may remain to condition the surface. However, if the increased adhesion exhibited by the LB grown cells was due to modification of the surface by adsorption of media components, then the effect should be uniform irrespective of growth stage. The data shows that attachment levels were significantly higher for late exponential phase cells i.e. these cells could attach more readily to a conditioned surface than stationary phase cells and therefore produced the high attachment ratios observed.

The trend of attachment ability through the growth phase appears to be similar whatever the media; attachment ratios were highest in late exponential phase cells.

There are contradictory reports in the literature as to the effect of growth phase; adhesion has been reported to increase in the exponential phase (Fletcher, 1977; Marshall *et al.*, 1971a) whilst others found decreased attachment in this phase (Kjelleberg and Hermansson, 1984). McEldowney and Fletcher (1988a) found that the levels of adhesion of a gliding organism, *Flexibacter*, were reduced in the stationary phase. They also found that the extent of attachment of *P.fluorescens* was independent of growth phase, whilst *Enterobacter cloacae* and *Chromobacterium* species showed lowest levels of attachment in the exponential phase with an increase in the stationary phase. It was concluded that the different levels of adhesion with growth phase was probably due to cell surface changes and was not dependant solely upon physiological activity.

Figure 4.7

Effect of growth phase and medium on the attachment of *S.liquefaciens*



S.liquefaciens was grown in the media specified at pH 7.5 at 25°C. Samples were removed at intervals for Optical density measurements, and cells were harvested and resuspended in buffer for the attachment assay (section 2.13). The number of attached cells was enumerated by direct epifluorescent microscopy and attachment ratios calculated. Triplicate stainless steel discs were used and 20 fields/disc were counted.

However the data presented in this study showed decreased activity during the stationary phase suggesting that physiological activity may in fact play an important role.

Marshall *et al.* (1971a) found that adsorption of logarithmic phase cells was more rapid than older cells. Similarly, Fletcher (1977) found that the highest proportion of motile cells were found in the exponential phase, and it was suggested that this may be linked to the increase in attachment ability exhibited by logarithmic cells. Stanley (1983) also found that logarithmic cells attached more readily than stationary phase cells and concluded that this might be due to a change in the surface of the stationary phase cells that reduced their ability to attach, but was not related to flagella expression. The effect of growth phase and nutrient limitation on polysaccharide production, outer membrane protein expression, hydrophobicity and charge are evaluated in subsequent chapters.

4.3.4 Effect of anaerobiosis on attachment abilities of *S.cohnii* and *S.liquefaciens*

Figure 4.8 shows the attachment ratios of *S.liquefaciens* and *S.cohnii* grown aerobically and anaerobically. (*P.fragi* is an obligate aerobe). The cell yield was considerably reduced in anaerobically grown cultures, however, cells were washed and adjusted to approximately the same concentration and the calculation of attachment ratios allowed for slight differences. As differences in attachment are observed in the different growth phases, the attachment of stationary phase aerobically and anaerobically grown cells were compared.

The results show that anaerobiosis had different effects on the attachment abilities of the two organisms. *S. liquefaciens* attached in higher numbers when aerobically grown, whereas *S.cohnii* attached at higher levels when grown anaerobically (approximately 3x higher attachment ratio).

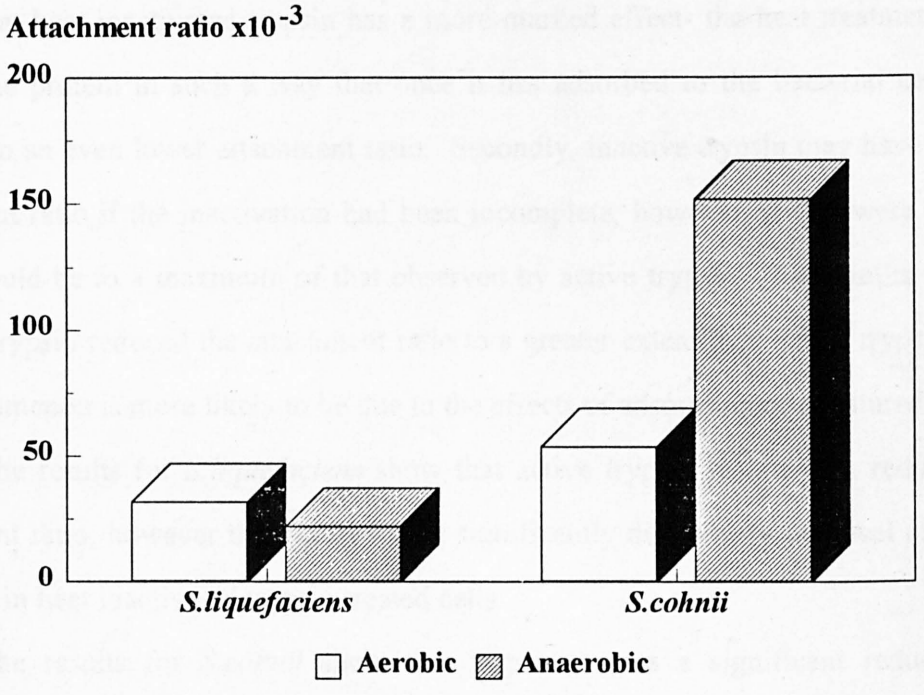
The differences in attachment abilities of aerobically and anaerobically grown cultures may be due to changes in several surface characteristics e.g. certain outer membrane proteins are expressed specifically under anaerobiosis (Lee and Falkow, 1990). However, other characteristics such as flagella expression, polysaccharide production, charge and hydrophobicity may be involved. *S.liquefaciens* is motile, and a higher level of aerobically

grown cells appeared to be motile by phase contrast microscopy as compared to an anaerobic culture. Higher levels of attachment were observed in the aerobic culture, but the fact that cells still attached without flagella shows that flagella are not necessary for attachment but may facilitate adhesion. The mechanism for this interaction may be by bridging/ overcoming the electrostatic repulsive barrier or alternatively increasing the statistical probability of collisions between the cell and the surface.

The effect of anaerobiosis of hydrophobicity, charge, outer membrane proteins, and polysaccharide production are discussed in subsequent chapters.

Figure 4.8

Effect of anaerobic growth on the attachment of *S.liquefaciens* and *S.cohnii*



Cultures were grown aerobically or anaerobically (section 2.5) in carbon and excess medium at 25°C, harvested cells were resuspended in buffer to a standard cell density. The number of cells attached to stainless steel after one hour was determined by direct epifluorescence microscopy and attachment ratios calculated (section 2.14). Triplicate stainless steel discs were used and 20 fields/disc were counted.

4.3.5 Effect of modification of the outer cell proteins on attachment ability.

Figure 4.9 shows the effect of trypsin on the attachment ratios of *S.cohnii*, *S.liquefaciens* and *P.fragi*. Cells were incubated in a trypsin suspension for one hour at 37°C to allow proteolytic modification of the surface proteins. The viability of the cells was determined before and after contact with the trypsin by TVC analysis, and showed that a contact time of one hour did not affect the viability of the cells.

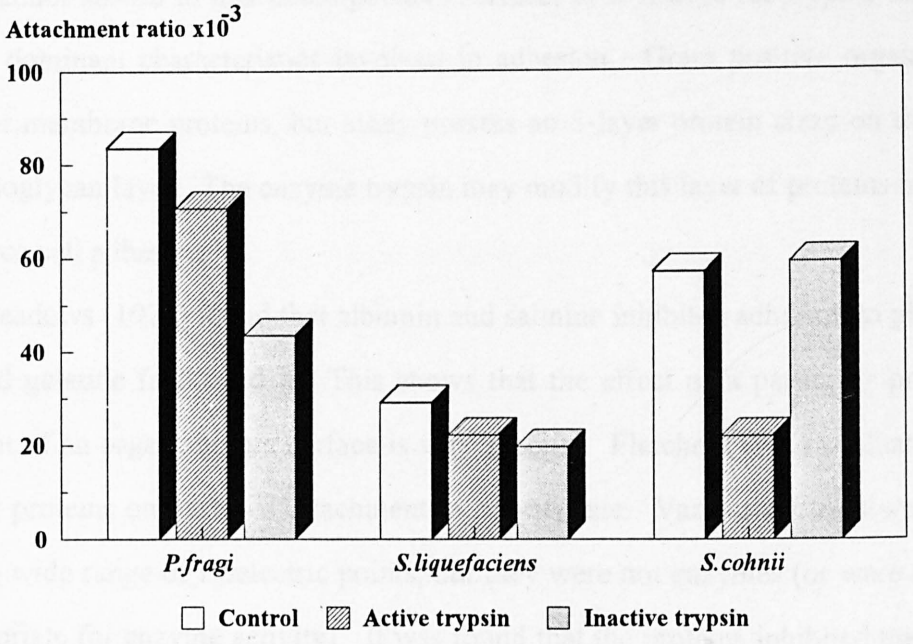
The data does not show consistent trends between the organisms. The attachment ratio of *P.fragi* is reduced by the action of the enzyme, but the attachment ratio of *P.fragi* treated with heat inactivated trypsin was even lower. This suggests two possibilities; firstly that although the cells were washed before the attachment assay, protein had adsorbed to the surfaces of the organism and this *per se* resulted in reduced attachment ability. It would appear that heat inactivated trypsin has a more marked effect- the heat treatment may have altered the protein in such a way that once it has adsorbed to the bacterial cell surface it resulted in an even lower attachment ratio. Secondly, inactive trypsin may have reduced the attachment ratio if the inactivation had been incomplete, however, if this were the case the effect would be to a maximum of that observed by active trypsin. The results showed that inactive trypsin reduced the attachment ratio to a greater extent than active trypsin, therefore the phenomenon is more likely to be due to the effects of adsorption of denatured protein.

The results for *S.liquefaciens* show that active trypsin results in a reduction in the attachment ratio, however this figure in not significantly different to the level of attachment observed in heat inactivated trypsin treated cells.

The results for *S.cohnii* show that trypsin causes a significant reduction in the attachment ratio (approximately half) as compared to the control, untreated cells. In the case of this organism, the inactive trypsin produced an attachment ratio similar to the control. Consequently it can be concluded that trypsin modified the cell surface in such a way as to significantly reduce the attachment ability of the organism. The fact that heat inactivated trypsin gave a similar result to the control untreated cells confirms that the inactivation was effective.

Figure 4.9

Effect of trypsin modification of the cell surface on attachment



Washed cells were resuspended in buffer, 1% (w/v) trypsin or 1% (w/v) heat inactivated trypsin for 1 hr at 37°C, the cells were then washed, adjusted to a standard cell density and added to the stainless steel surfaces. Triplicate discs were used and 20 fields/disc were counted. The number of attached cells was determined by direct epifluorescent microscopy, and attachment ratios calculated.

It may be suggested that the effect that inactive trypsin had on *P.fragi* and *S.liquefaciens* was through adsorption to the cell surface thereby modifying the attachment ability of the organisms, as inactive trypsin had no effect on *S.cohnii* it would seem that the denatured enzyme cannot adsorb to this Gram positive surface, or if it does the trypsin does not mask the more dominant characteristics involved in adhesion. Gram positive organisms do not have outer membrane proteins, but many possess an S-layer protein array on the outside of the peptidoglycan layer. The enzyme trypsin may modify this layer of proteins in such a way as to reduce cell adhesion.

Meadows (1971) found that albumin and salmine inhibited adhesion to glass, whereas casein and gelatine facilitated it. This shows that the effect of a particular protein on the attachment of an organism to a surface is very specific. Fletcher (1986) evaluated the effect of several proteins on bacterial attachment to polystyrene. Various proteins were examined to cover a wide range of isoelectric points, but they were not enzymes (or were not used at a pH appropriate for enzyme activity). It was found that the proteins inhibited the attachment, although only one protein, bovine serum albumin, was thought to inhibit by adsorbing to the bacterial cell surface, whilst the other proteins were thought to modify the substratum. This data ties in with the results obtained with inactive trypsin, which produced reduced levels of attachment of *P.fragi* and *S.liquefaciens.*

The observations were evaluated in more detail by examination of the outer membrane proteins, hydrophobicity and lipopolysaccharides present after trypsin treatment and are discussed in later chapters.

4.4 Development of a system to model biofilm formation

Various systems have been used to examine biofilm development from simple static attachment systems (as in section 4.3) with no flow, to complex reactor systems. In batch systems the concentration of substrates and products obviously vary with time. Continuously fed stirred tank reactor systems or chemostats are constant volume open systems. The system attains a steady state due to the constant addition of nutrients and the constant overflow. Another system is the plug flow reactor where reactants are fed at a continuous rate to the reactor thus establishing at any position between the inlet and outlet a steady state composition (Bryers and Characklis, 1992).

A large number of studies have been conducted using standard laboratory fermenter vessels adapted to allow periodic sampling of biofilms. Usually the surfaces are introduced through existing sample ports but the number of samples is limited. Molin and Nilsson (1983) used this type of system to examine the accumulation of a *Pseudomonas putida* biofilm. Cowan *et al.* (1991) used a similar system to examine the colonisation of glass coverslips by *Aeromonas hydrophila, Pseudomonas fluorescens, Xanthomonas maltophila* and a coryneform.

Rotating annular reactors have been used by some workers (Trulear and Characklis, 1979; Banks and Bryers, 1991; Christensen *et al.*, 1989). This comprises two concentric cylinders fitted one inside the other separated by a small gap. One cylinder remains stationary and the other is free to move. Removable test plugs are engineered into the outer cylinder.

Flow cell reactors (tubular flow and parallel plate flow cells) have been used to model biofilm development in a wide range of environments (Bryers and Characklis, 1992).

Fowler and McKay (1980) described a radial flow reactor which consisted of two parallel circular discs separated by a small gap (500 μm) which was used to examine the fluid velocities that inhibit biofilm formation.

Other systems that have been used to model biofilm development include the constant depth laboratory film fermenter (Peters and Wimpenny, 1988) and the perfused filter system of Gilbert *et al.* (1989).

The system used in this work was a modification of a standard laboratory chemostat. A 2l chemostat was used to grow a steady state population of either single or mixed species. A range of dilution rates and therefore growth rates were used between 0.02-0.22 hr^{-1} but were all below μmax. Slow growth rates were chosen, since the food factory environment presents the organisms with various stresses and limitations and consequently may be growing very slowly. Section 2.6 describes the operating conditions of this system. The unit was run at 25°C, close to the ambient temperature in many factories. Sections 4.4.1 and 4.4.2 describe the results obtained using single and tri-species populations.

Once a steady state was achieved, the surface chamber was introduced to the chemostat on a loop (see section 2.12 for a description). In order for the system to be operated as a continuously fed, stirred tank reactor the recycle flow rate to the surface vessel was much greater than the nutrient feed flow rate. Preliminary studies confirmed that the degree of mixing was effective and that there was no difference between cell and oxygen concentrations in the chemostat and the surface vessel. The surface vessel held up to forty-two 13 mm diameter stainless steel discs, and initial studies showed that there was no difference in attachment at different positions in the chamber.

Sections 4.5.1 and 4.5.2 describe the attachment data obtained using this system. Section 4.5.3 describes studies examining the stability of the mixed population and section 4.5.4 examines the differences in activity between liquid phase and attached cells.

4.4.1 Mixed culture chemostat studies

4.4.1.1 Carbon limited

Figures 4.10-4.14 show the liquid phase populations in a carbon limited chemostat at different dilution rates. The points on the graphs shown are means of triplicate samples from two or three runs of the same experiment.

The dominant organisms at 0.017 hr^{-1} (Fig 4.10) were *S.liquefaciens* and *P.fragi* which were present at similar levels with *S.cohnii* present at a negligible level. At 0.05 hr^{-1} (Fig 4.11) *S.liquefaciens* was the dominant organism, followed by *P.fragi* and the *S.cohnii*. There is little difference between the population produced at this dilution rate and the higher

rates other than that the steady state level of *S.cohnii* is higher - 10^5 cells ml^{-1}. At 0.075 hr^{-1} (Fig 4.12), *S.liquefaciens* was again the dominant organism but the difference between *S.liquefaciens* and *P.fragi* was greater (approximately 1-2 log orders). *S.cohnii* reached steady state around 10^4 cells ml^{-1}.

Fig 4.13 shows that at 0.133 hr^{-1} the trend was similar but the difference between *S.liquefaciens* and *P.fragi* was greater. *P.fragi* and *S.cohnii* reached steady state levels around 10^6 and 10^4 cells ml^{-1} respectively.

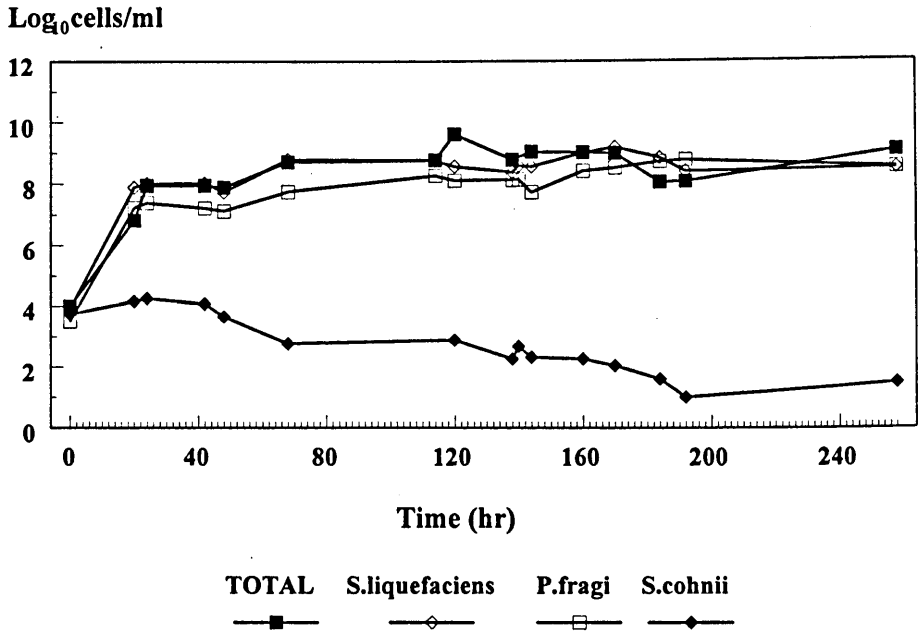
At the highest dilution rate (Fig 4.14) the differences between the steady state populations was most marked, with *S.liquefaciens* reaching 10^9 cells ml^{-1}, *P.fragi* around 10^7 cells ml^{-1} and *S.cohnii* 10^5 cells ml^{-1} at steady state.

The general trend was that at all dilution rates *S.liquefaciens* was the dominant organism. The difference in steady state concentration of *S.liquefaciens* and the other two organisms increased with increasing dilution rate. This would suggest that the associated increase in growth rate is applicable only to *S.liquefaciens.* Competition for the limiting substrate will mean that although the system is carbon limited, the limitation of the substrate for the organisms that are less competitive will be greater and may consequently have lower growth rates. The aim of this work was to produce a stable mixed culture population to be used in studies of attachment to stainless steel surfaces. The data presented indicates that the population is relatively stable from 20 hr up to 240 hr.

The total steady state cell concentration remained relatively constant around 10^8 cells ml^{-1}, although at 0.133 hr^{-1} and 0.217 hr^{-1} the concentration is slightly higher around 10^9 cells ml^{-1}. The system was run routinely below μmax and therefore changes in cell density would be expected to remain relatively constant irrespective of growth rate over the range studied.

Figure 4.10

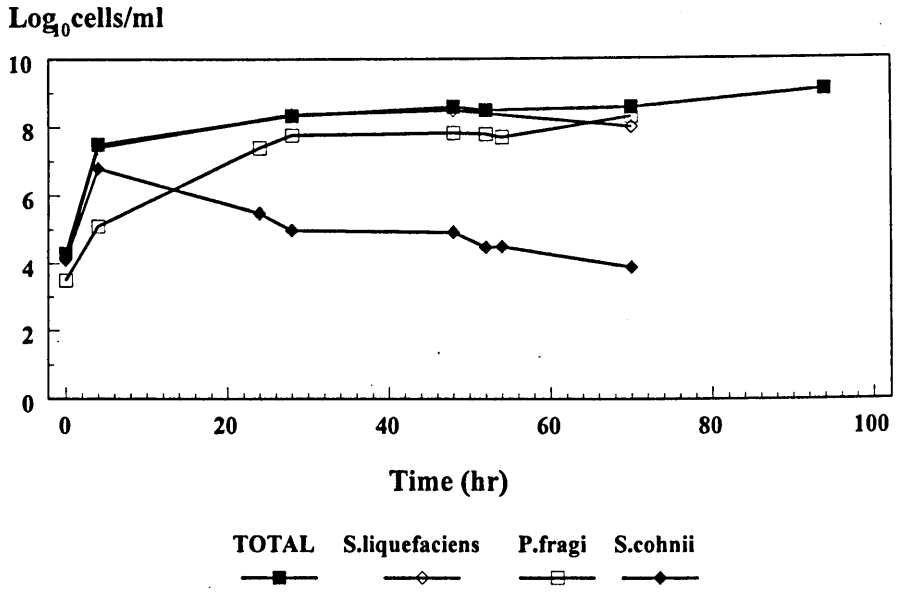
Carbon limited mixed culture chemostat at 0.017 hr^{-1}



The bacteria were inoculated into the carbon limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of two runs at this dilution rate.

Figure 4.11

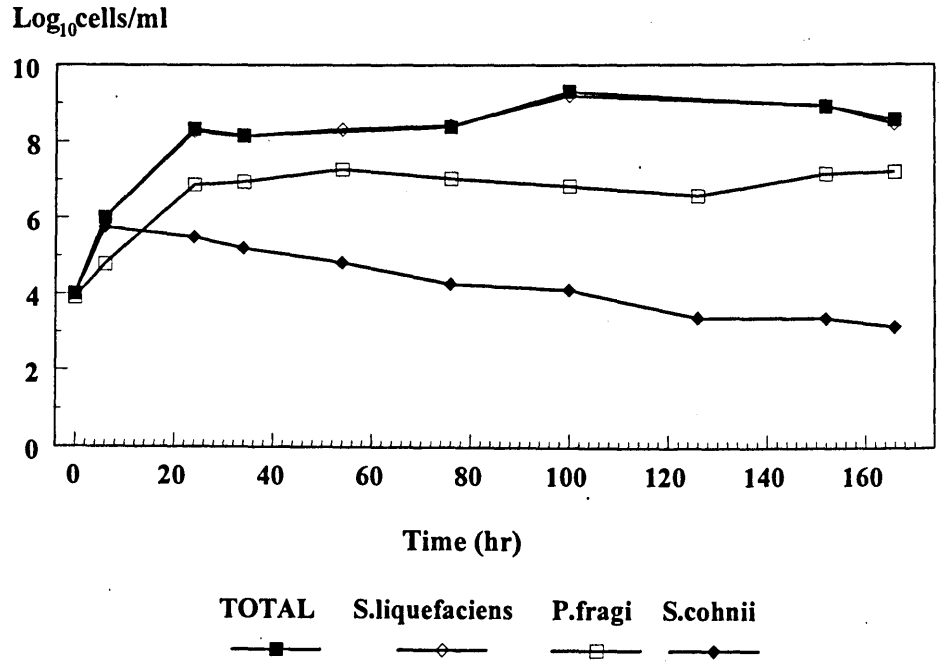
Carbon limited mixed culture chemostat at 0.05 hr^{-1}



The bacteria were inoculated into the carbon limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of three runs at this dilution rate.

Figure 4.12

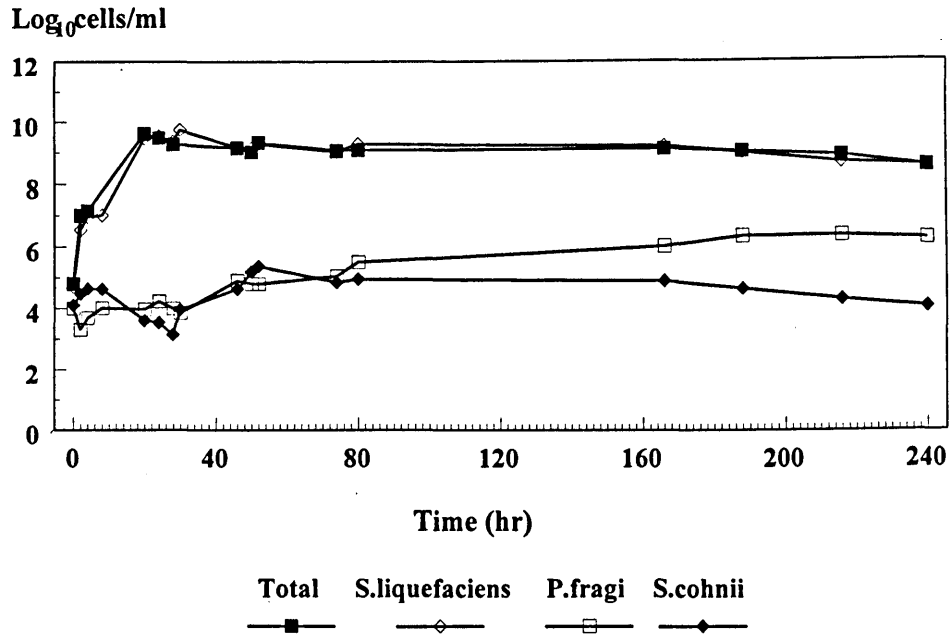
Carbon limited mixed culture chemostat at 0.075 hr^{-1}



The bacteria were inoculated into the carbon limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of two runs at this dilution rate.

Figure 4.13

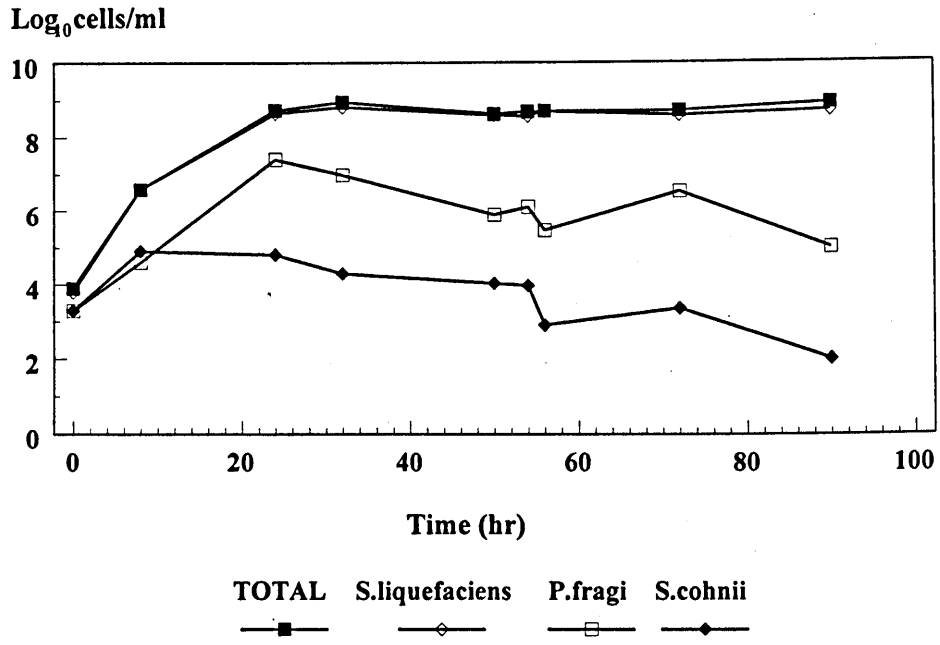
Carbon limited mixed culture chemostat at 0.133 hr^{-1}



The bacteria were inoculated into the carbon limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of three runs at this dilution rate.

Figure 4.14

Carbon limited mixed culture chemostat at 0.217 hr^{-1}



The bacteria were inoculated into the carbon limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of two runs at this dilution rate.

4.4.1.2 Nitrogen limited

Figures 4.15-4.17 show the liquid phase concentrations of the three organisms under nitrogen limitation. At 0.075 hr^{-1} the concentration of *S.liquefaciens* and *P.fragi* were very similar at steady state (approximately 10^8 cells ml^{-1}) (Figure 4.15). *S.cohnii* did not grow readily under nitrogen limitation and required the addition of a mixture of growth factors (guanine, pyridoxine, tyrosine, sodium thiosulphate, prolin and histidine). This was reflected in the fact that the steady state level of this organism was low.

Figure 4.16 shows that a similar trend was observed at 0.133 hr^{-1}, but the level of *S.liquefaciens* appeared to be slightly higher than for *P.fragi.* The level of *S.cohnii* was slightly higher around 10^4 cells ml^{-1}, but this organism was gradually lost from the system. The total steady state level was approximately 10^8 cells ml^{-1} and therefore similar to that observed at 0.075 hr^{-1}.

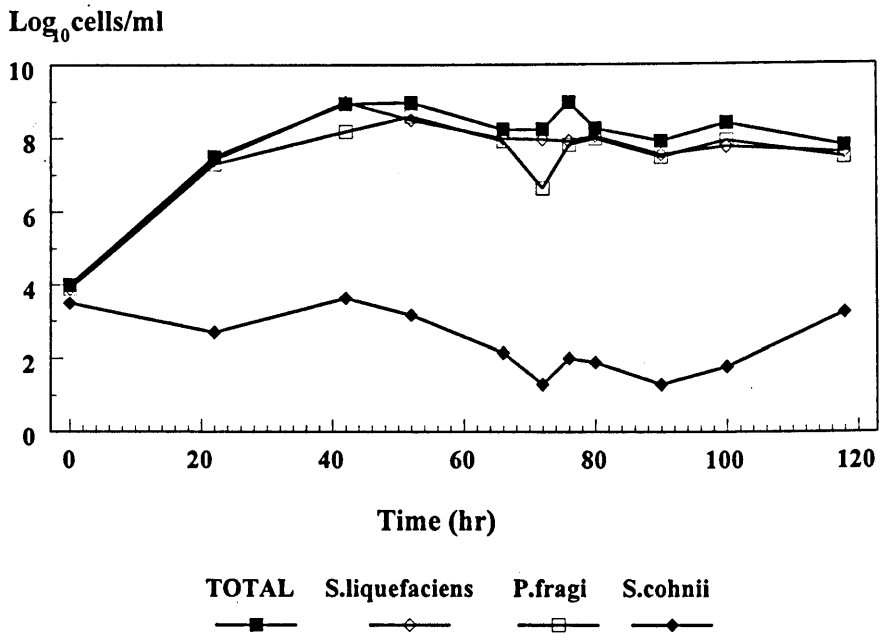
At 0.217 hr^{-1} (Figure 4.17), the loss of *S.cohnii* was more marked as the population reduced from 10^4 cells ml^{-1} to less than 10^2 cells ml^{-1} over approximately 70 hr. The levels of *S.liquefaciens* and *P.fragi* were again similar with *S.liquefaciens* slightly higher. The total steady state cell concentration was similar.

In contrast to carbon limitation, where there were obvious differences between the steady state concentrations of the three organisms, under nitrogen limitation the level of *S.liquefaciens* and *P.fragi* were similar, and the levels of *S.cohnii* were lower under nitrogen limitation. Dilution rate appeared to have less effect on the populations under nitrogen limitation than under carbon limitation. The steady state was maintained from 20-100 hr, although the levels of *S.cohnii* gradually reduced during this period.

It was concluded that the three species could be grown together in the chemostat reproducibly and that for a specified time a relatively stable steady state population was achieved.

Figure 4.15

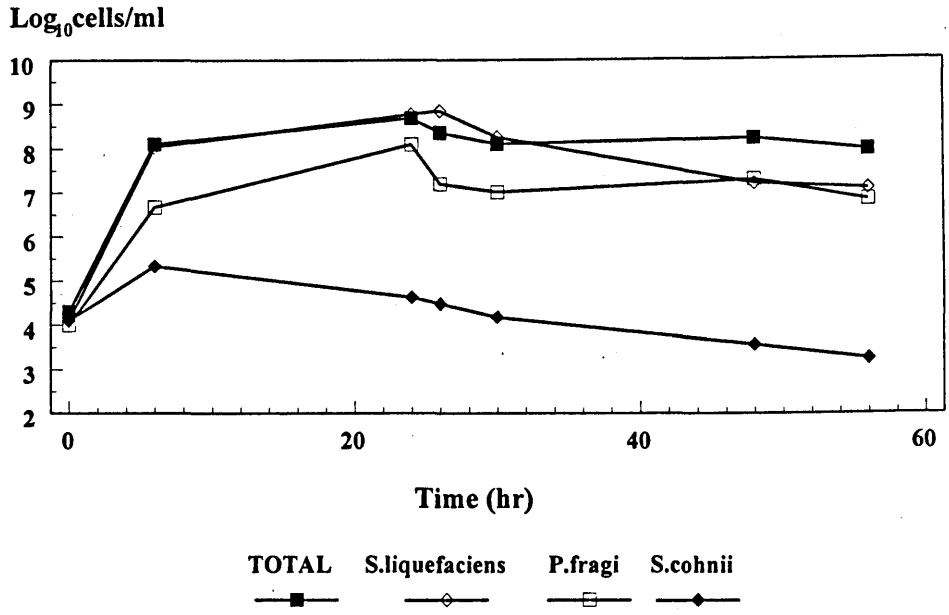
Nitrogen limited mixed culture chemostat at 0.075 hr^{-1}



The bacteria were inoculated into the nitrogen limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of two runs at this dilution rate.

Figure 4.16

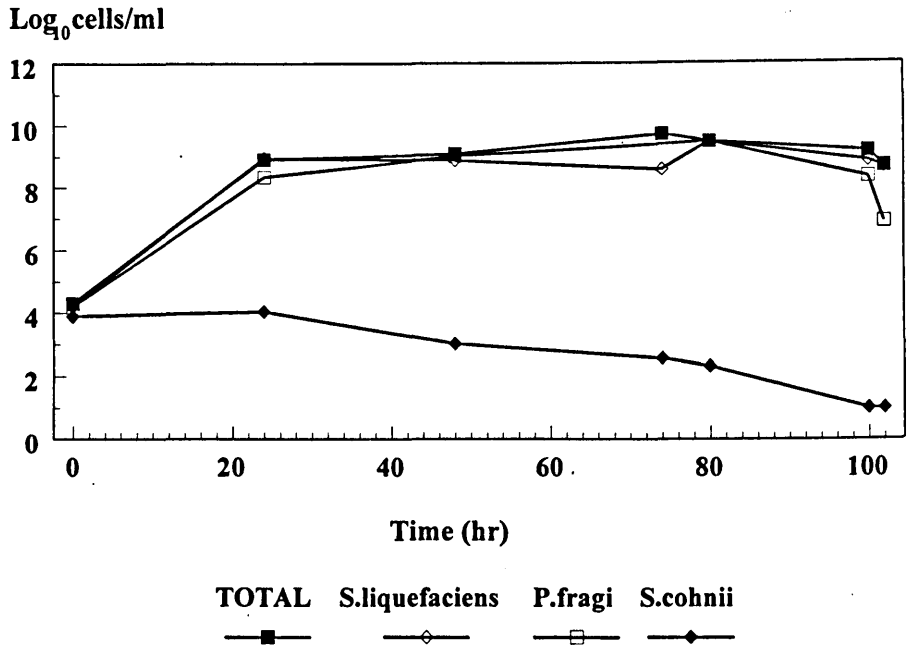
Nitrogen limited mixed culture chemostat at 0.133 hr^{-1}



The bacteria were inoculated into the nitrogen limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of three runs at this dilution rate.

Figure 4.17

Nitrogen limited mixed culture chemostat at 0.217 hr^{-1}



The bacteria were inoculated into the nitrogen limited chemostat at t=0 and samples were removed at intervals for serial dilution and plating onto selective agars to quantify the three species (section 2.7). The counts are means of two runs at this dilution rate.

4.4.2 Monoculture chemostat studies

Figures 4.19-4.21 show the steady state populations achieved by the three organisms in carbon limited monoculture. The results show that the steady state cell density of the monocultures was similar to the total cell density in the mixed species chemostat. The fact that *P.fragi* and *S.cohnii* were present at higher levels in monoculture shows that these two organisms were further limited in the mixed culture chemostat by *S.liquefaciens* either by production of growth inhibitors or more likely through competition for the growth substrates.

4.5 Attachment and biofilm development

The surface vessel was introduced to the chemostat once steady state had been achieved. Surfaces were removed at intervals, and the total number of cells on the surfaces was determined by acridine orange staining (Figure 4.18A) and direct epifluorescent microscopy and the individual species were quantified using indirect immunofluorescent labelling (Figure 4.18B). Surfaces were also removed for critical point drying and scanning electron microscopy.

4.5.1 Attachment from monoculture

Figure 4.19 shows the attachment of *S.cohnii* to stainless steel from a steady state monoculture. The results show that there was a rapid increase in the concentration of cells on the surface which stabilized at 10^7 cells cm^{-2}.

The attachment of *S.liquefaciens* to stainless steel is shown in Fig 4.20 and shows a similar trend, although the maximum level achieved was lower than that produced by *S.cohnii* even though the liquid phase steady state concentration was significantly higher. This observation is reflected in the attachment ratios, as *S.liquefaciens* had a low attachment ratio compared to *S.cohnii.*

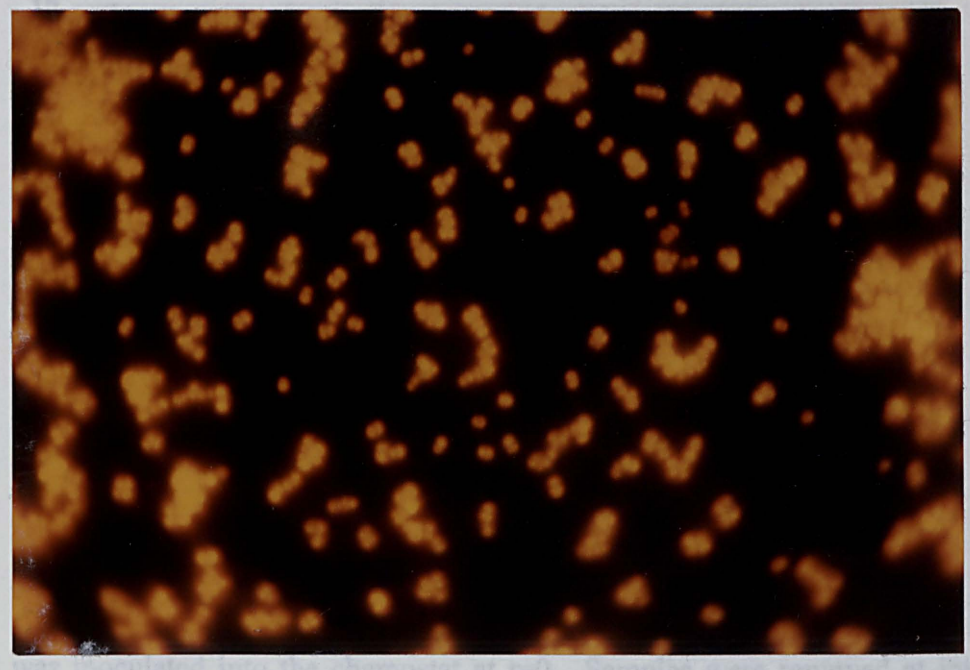
Figure 4.21 shows the attachment of *P.fragi.* This organism attached in higher numbers than *S.liquefaciens* but was similar to *S.cohnii.*

Although there are some differences in the liquid phase concentrations this does not explain the difference in levels of attachment as the organism with the lowest liquid phase concentration produced the highest level of cells on the surfaces. Nutrients are thought to accumulate at interfaces, therefore, there may be a higher concentration of nutrients at the surface. Consequently, the micro-environment of the surface may be very different to the nutrient limitations experienced in the bulk liquid phase and therefore it would be advantageous to the cell to be attached to the surface. The following section (section 4.6) examines the attachment of the bacteria from a mixed culture. Subsequent chapters examine factors that may be involved in attachment and subsequent biofilm development in order to explain why the three species have different attachment abilities.

Figure 4.18

Acridine orange and immunofluorescent labelling of attached cells

A. Acridine orange stained *S.cohnii* (Magnification 1000x)



B. Immunofluorescent labelling of the three species mixed population (Magnification 1000x)

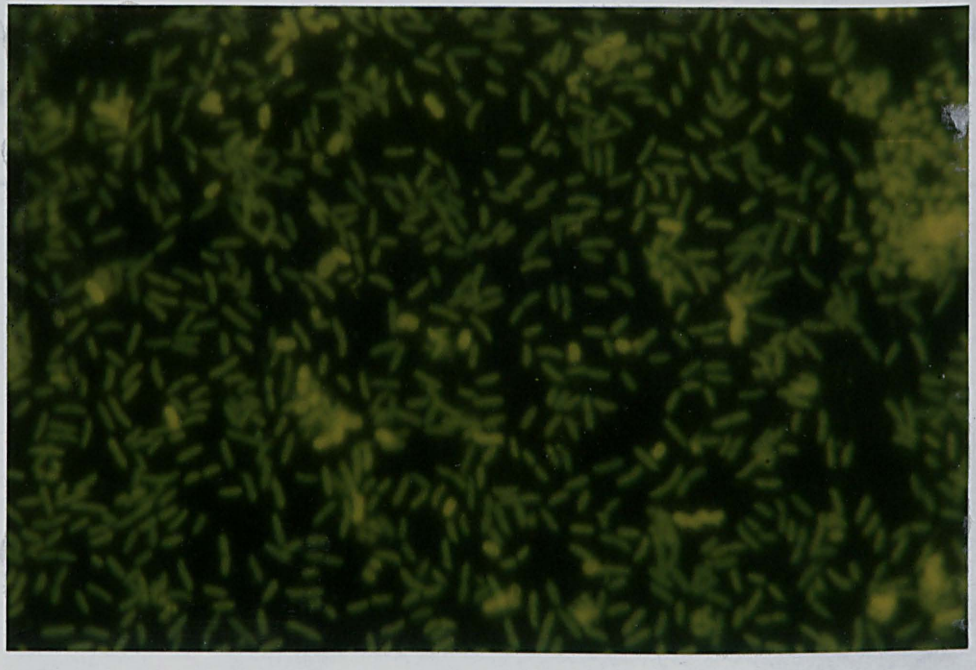
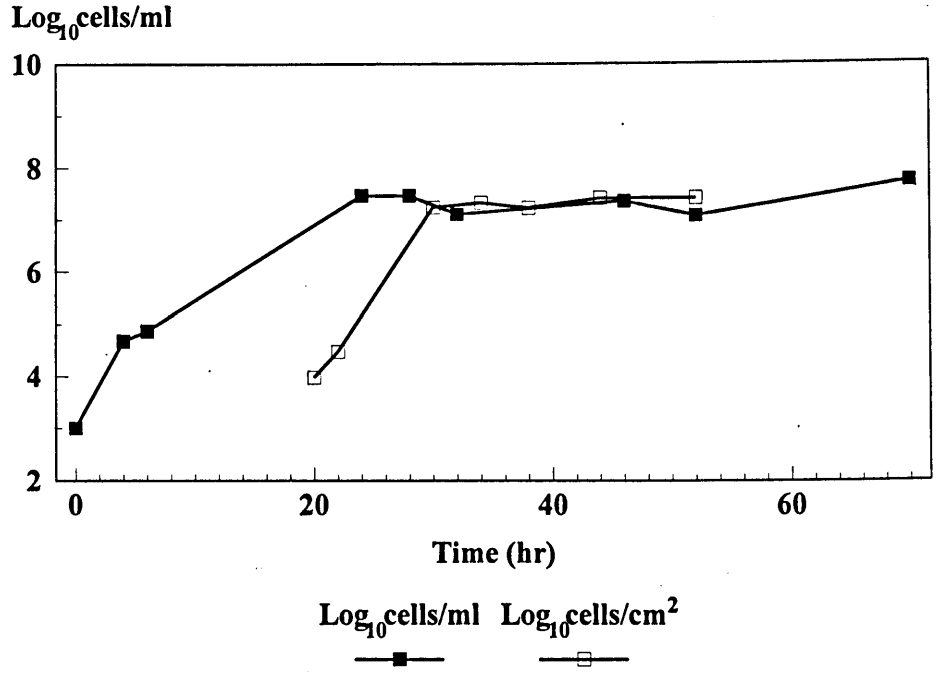


Figure 4.19

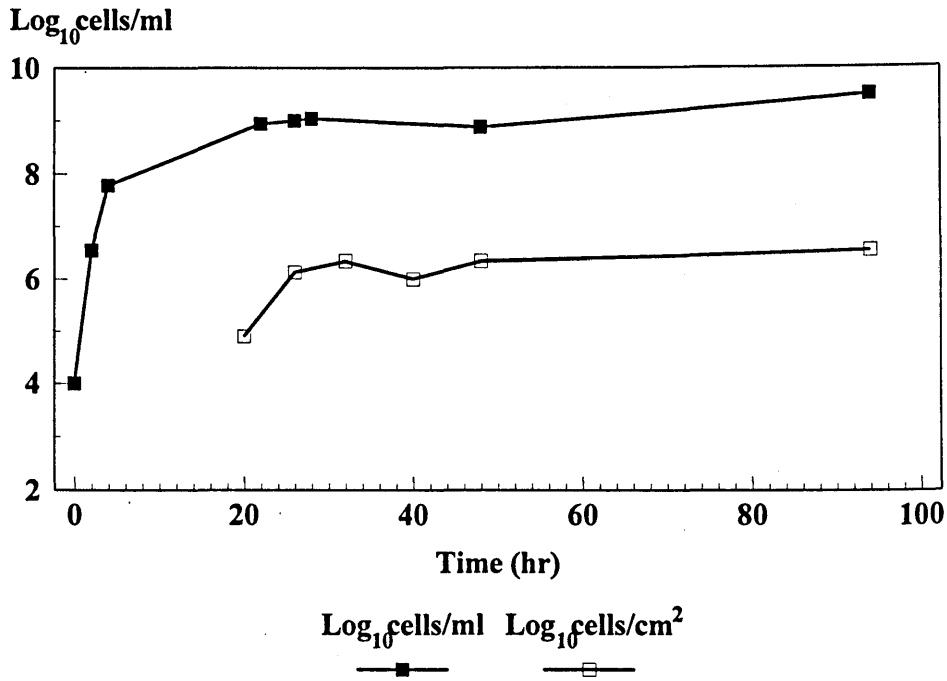
Attachment of *S.cohnii* to stainless steel from carbon limited monoculture (dilution rate 0.02 hr^{-1})



S.cohnii was inoculated into the chemostat at t=0. Samples were removed at intervals for viable count determinations (section 2.7). The surface vessel was introduced to the system at steady state, and triplicate stainless steel discs were removed at intervals for direct epifluorescent counts (section 2.14).

Figure 4.20

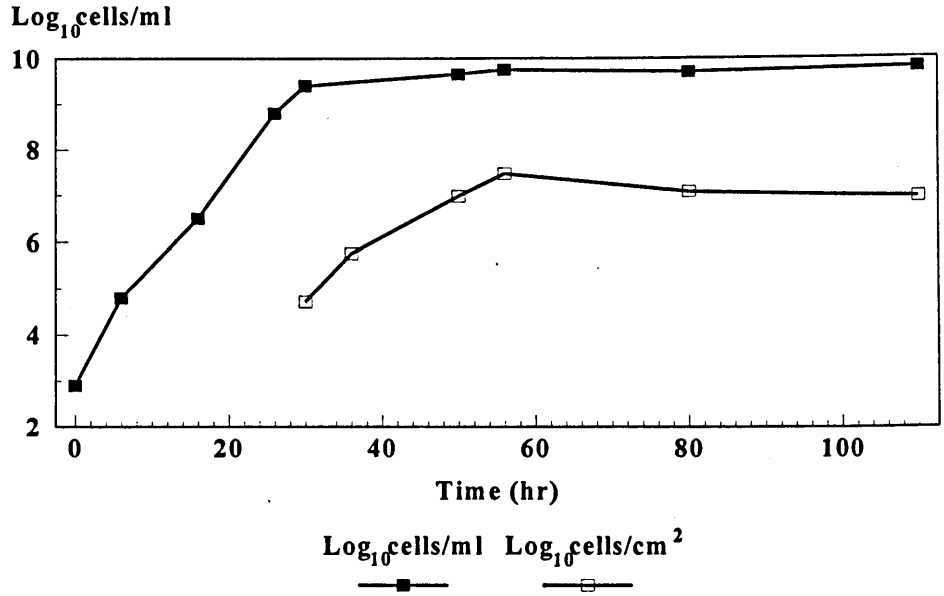
Attachment of *S.liquefaciens* to stainless steel from a carbon limited culture at 0.02 hr^{-1}



S.liquefacuens was inoculated into the chemostat at t=0. Samples were removed at intervals for viable count determinations (section 2.7). The surface vessel was introduced to the system at steady state, and triplicate stainless steel discs were removed at intervals for direct epifluorescent counts (section 2.14).

Figure 4.21

Attachment of *P.fragi* to stainless steel from carbon limited monoculture at 0.02 hr^{-1}



P.fragi was inoculated into the chemostat at t=0. Samples were removed at intervals for viable count determinations (section 2.7). The surface vessel was introduced to the system at steady state, and triplicate stainless steel discs were removed at intervals for direct epifluorescent counts (section 2.14).

Biofilm development appeared to be limited as 10^7 cells cm^{-2} was calculated as approaching a monolayer of attached cells. However, the chemostat was subjected to a very slow dilution rate of 0.02 hr^{-1} and a multilayered biofilm may take longer to develop. Studies by Holah and Kearney (1992) concluded that in food factory environments the biofilms that developed were not generally multilayered films but monolayers due to the relatively short time available for biofilm development in this type of environment. Consequently the monolayer biofilm developed in this work may in fact be a reasonable representation of factory environment biofilms.

4.5.2 Attachment from mixed cultures

4.5.2.1 Carbon limited chemostat

The attachment ratios for the three organisms from a carbon limited chemostat at different dilution rates are shown in Table 4.1. Attached cells effectively become trapped in the system and are not subject to the wash out from the chemostat experienced by the liquid phase cells, consequently they remain in place as fresh nutrients are added and therefore growth rate control is only effected against the liquid phase cells.

At 0.02 hr^{-1} *S.liquefaciens* had the lowest attachment ratios. The attachment ratio was calculated by dividing the concentration of cells on the surface by the liquid phase cell concentration. Therefore a low attachment ratio for *S.liquefaciens* means that although this organism had a high concentration in the liquid phase, it was present in relatively fewer numbers on the surface. *S.liquefaciens* was still the dominant organism of the surface population, however it was present as a smaller proportion of the population compared to its composition of the liquid phase. *S.cohnii* and *P.fragi* have relatively high attachment ratios i.e. these organisms make up a higher proportion of the surface population than of the liquid phase population. The general increase in cell numbers over time reflects the increase in cell numbers on the surface with respect to the liquid phase concentration which remained relatively stable.

A similar trend is observed at 0.133 hr^{-1} as *S.liquefaciens* had the lowest attachment ratio, and *S.cohnii* the highest. The attachment ratio for *P.fragi* was lower at 0.133 hr^{-1} than 0.02 hr^{-1}.

Similarly, at 0.217 hr^{-1}, *S.cohnii* had the highest attachment ratios and *S.liquefaciens* the lowest. It appears that the organism with the highest growth rate (although the organisms were grown in a chemostat the rapid utilization of the growth substrate by the dominant organism means that the other two organisms were restricted) had the lowest attachment ability, and the organism that performed least well in the liquid phase was relatively better at surface colonization.

Table 4.1

Attachment ratios from a carbon limited fermenter at different dilution rates

Dilution rate (hr^{-1})	Time (hr)	*S.liquefaciens*	*P.fragi*	*S.cohnii*
0.02	2	11.68	206	511
	4	4.5	311	451
	24	6.9	241	6627
	47	34.9	694	None detected
0.133	2	5.0	27.8	1232
	4	5.4	48.5	1035
	6	10.4	66.5	1063
	8	4.3	105.6	1800
	24	16.7	326	None detected
0.217	2	1.14	991.3	6827
	4	2.04	755.2	6322
	6	1.00	915.4	None detected
	8	2.88	5133.3	None detected
	24	1.45	1041.1	None detected

Figures in the table are attachment ratios x 10^{-3}. Surfaces were added to the chemostat system once steady state had been reached, and the times refer to time after addition of the surfaces. The different species were quantified using indirect immunofluorescent labelling.

Attachment to a surface may provide an ecological advantage, for example nutrients are thought to be concentrated at the liquid surface interface which will be particularly important in low nutrient environments. In the chemostat system *S.cohnii* in particular was subject to nutrient limitations due to the rapid metabolism of *S.liquefaciens* and therefore the surface environment may be more favourable.

At 0.133 hr^{-1} both *S.liquefaciens* and *P.fragi* and at 0.217 hr^{-1} *P.fragi* showed increases in attachment ratios with time. This reflects an increase in number of organisms on the surface of these particular organisms as the liquid phase level is at steady state.

In conclusion, the results show that dilution rate did not affect the relative attachment abilities of the three organisms over the range tested, *S.cohnii* had the highest ratio, then *P.fragi* and *S.liquefaciens* the lowest. Increasing the dilution rate appeared to decrease the levels of *S.liquefaciens* on the surface, but had the opposite effect on levels of *S.cohnii.* The effect of dilution rate on the levels of attachment of *P.fragi* was less clear. McEldowney and Fletcher (1986) found that dilution rate had varied effects on cell attachment depending on the organism; *Flexibacter* and *Chromobacterium* sp both showed increased attachment with increasing dilution rate whilst *P.fluorescens* and *Enterobacter cloacae* were relatively unaffected by dilution rate.

4.5.2.2 Nitrogen limited

The attachment ratios for the three organisms from a nitrogen limited chemostat at different dilution rates are shown in Table 4.2. At 0.075 hr^{-1} the trend observed under carbon limitation was noted, i.e. *S.cohnii* had the highest attachment ratio and *S.liquefaciens* and *P.fragi* lower. *S.cohnii* was not detected on the surfaces at the higher dilution rates, which would seem to suggest that *S.cohnii* was lost from the surface and due to loss from the chemostat system no longer detected. This may suggest a dynamic exchange of organisms between the surface and liquid phases.

Table 4.2

Attachment ratios from a nitrogen limited fermenter at different dilution rates

Dilution rate (hr^{-1})	Time (hr)	*S.liquefaciens*	*P.fragi*	*S.cohnii*
0.075	2	8.4	3.67	553.2
	4.5	7.97	13.9	692.1
	8.5	13.0	31.7	517.6
0.133	3	0.60	72.5	None detected
	6	6.60	173.0	None detected
	8	16.47	252.3	None detected
0.217	3	1.41	0.56	None detected
	6	1.23	0.43	None detected
	10	2.79	1.43	None detected

Figures refer to attachment ratios x 10^{-3}. Surfaces were added once steady state had been reached, and the times are time after addition of the surfaces to the chemostat system. The individual species were quantified by indirect immunofluorescent labelling.

P.fragi and *S.liquefaciens* had similar attachment ratios under nitrogen limitation, as figures 4.16 and 4.17 show these organisms were present in similar numbers under nitrogen limitation. This would suggest that they are equally successful in the competition for growth substrate, and therefore will have similar growth rates under these conditions. When the chemostat was operated with a mixed culture, different limitations are imposed on the system other than the designated carbon or nitrogen limitation. Consequently under carbon limitation *S.liquefaciens* was the dominant organism and out-competes the other two

organisms, whilst under nitrogen limitation *S.liquefaciens* and *P.fragi* performed equally well and may therefore be expected to have similar growth rates. If this is the case then the differences in attachment ability may relate to a growth rate effect. *S.cohnii* was the organism with probably the lowest growth rate under all conditions and this organism had the highest attachment ratios, except when the organism was lost from the system.

Banks and Bryers (1991) studied the net rate of accumulation, biofilm composition and removal rates using *Pseudomonas putida* and *Hyphomicrobium* sp. Although *Hyphomicrobium* sp produce a holdfast thought to be a specific adaptation for surface adhesion, a biofilm of this organism was rapidly colonized by *P.putida* resulting in the latter becoming the dominant organism. They found that the establishment of a depositing organism in a biofilm composed of the other organism was found to be a function of relative growth rates. In experiments similar to those conducted in this work, i.e. sterile surfaces challenged with a mixed culture, they found that the faster growing organism became the dominant biofilm species and the slower growing organism remained established within the biofilm. This compares favourably with the work presented here, as *S.liquefaciens* was the fastest growing organism and dominant in both the liquid and surface phases. However, the relative numbers of the slow-growing organism was higher on surfaces than in the liquid phase and when there was a change in culture conditions from carbon to nitrogen limitation, *P.fragi* did relatively better in the liquid phase and this was associated with a decrease in attachment potential.

Cowan *et al.* (1991) investigated the colonisation of glass by three or four bacterial species over 4 weeks in a recirculating model laboratory system. They monitored the liquid phase and surface populations over time and under different nutrient conditions, and found that the relative proportions of the species in the liquid phase and on the surface increased with increasing nutrient concentration but the effect was more marked in the liquid phase cells. This may reflect a difference in growth rates between the liquid and attached cells as suggested above.

As observed with carbon limitation, the attachment ratios generally showed an increase over time, reflecting the build up of organisms on the surfaces. As was observed

with single species attachment studies, the concentration of cells on the surface rapidly attained a relatively constant level, after which there was only a gradual increase in cell numbers on the surface.

Dilution rate had no obvious effect other than to increase the wash out of *S.cohnii* so that the numbers fell below detectable levels.

In natural environments, pure cultures are rare, and species may interact in such a way as to affect surface colonization through cell adhesiveness changes and consequently competitive success. Some studies have indicated that the attachment of one organism may be enhanced by the presence of another species. McEldowney and Fletcher (1987) showed that the attachment of *Acinetobacter* sp promoted the subsequent attachment of a *Staphylococcus* sp. Other cell interactions that promote adhesion are found in the oral cavity where the colonization of the tooth surface is by a characteristic assembly of coaggregative organisms (Kolenbrander, 1988). The attached cells of primary colonizers may condition the surface so that subsequent attachment is dependent on cell-cell interactions between organisms. These synergistic interactions may allow a poor colonizer to become established. In the case of the biofilm developed in this study, it may be advantageous for *S.cohnii* to be attached to a surface in the proximity of other organisms so that *S.cohnii* can metabolize the other cell by-products, polysaccharides, or scavenge the nutrients that may be concentrated at the surface by adsorption. Cowan *et al.* (1991) observed a similar phenomena with *Aeromonas hydrophila* which was rapidly lost from the liquid phase at low nutrient concentrations but showed an increase in numbers on the surfaces. These workers suggested that this may occur due to the development of anoxic conditions in areas of the biofilm, which *A.hydrophila* as a facultative anaerobe could exploit. This is a possibility in the system presented here, although the biofilm is not very thick, the centre of microcolonies may be subject to reduced levels of oxygen, and *S.cohnii* is also a facultative anaerobe. This is unlikely as *P.fragi,* an aerobe, attached readily and was found in relatively higher numbers on the surface than in the liquid phase.

An alternative explanation for the increased levels of *S.cohnii* and *P.fragi* on the surface may be due to differences in surface adhesiveness of the three organisms.

McEldowney and Fletcher (1986) found that different nutrient conditions and growth rates resulted in changes in the attachment abilities of the organisms, but the changes were organism specific. Various workers have observed an influence of nutrient condition on adhesiveness of several bacteria including *Pseudomonas putida* (Molin *et al.*, 1982) marine Pseudomonads (Marshall *et al.*, 1971a) and the oral *Streptococci* (Knox *et al.*, 1985). Dawson *et al.* (1981) observed adhesiveness of a marine *Vibrio* when incubated in a salt solution without a carbon or energy source. Cowan *et al.* (1991) also found increased adhesion under low nutrient conditions.

Subsequent chapters examine cell surface characteristics that may explain the differences in attachment ability and include hydrophobicity, charge, polysaccharide production, LPS and OMP expression under various growth conditions.

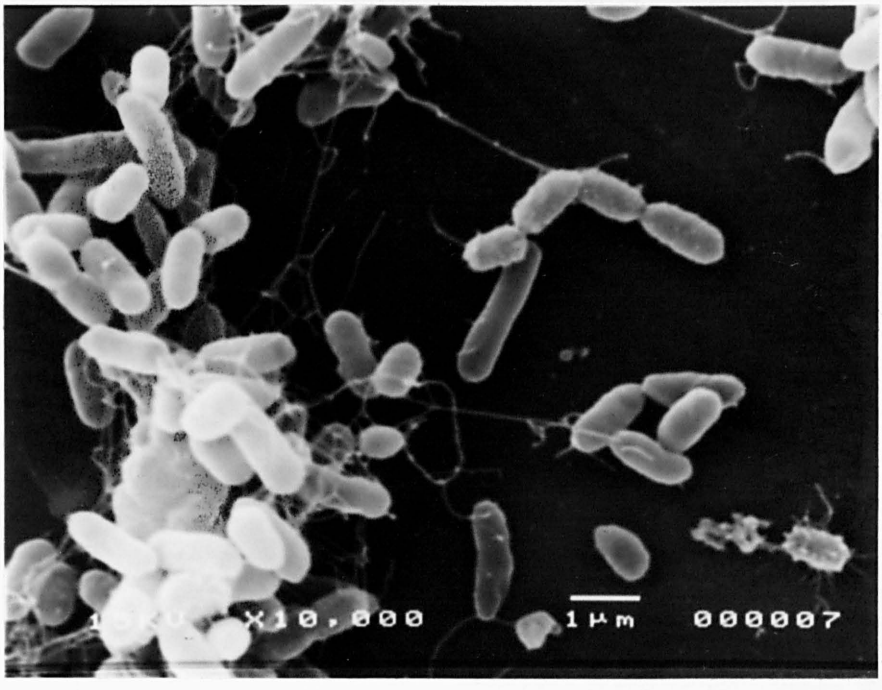
4.5.2.3 Scanning electron microscope examination of biofilm development

The micrographs shown in Figure 2.22 show the development of the mixed culture biofilm over a 24 hour period. The biofilm does not cover the surface completely, but appears to be made up of microcolonies which may be several cells deep. Polymeric material is visible between the cells and between the cells and the surface, and at 24 hours forms an extensive matrix covering the cells.

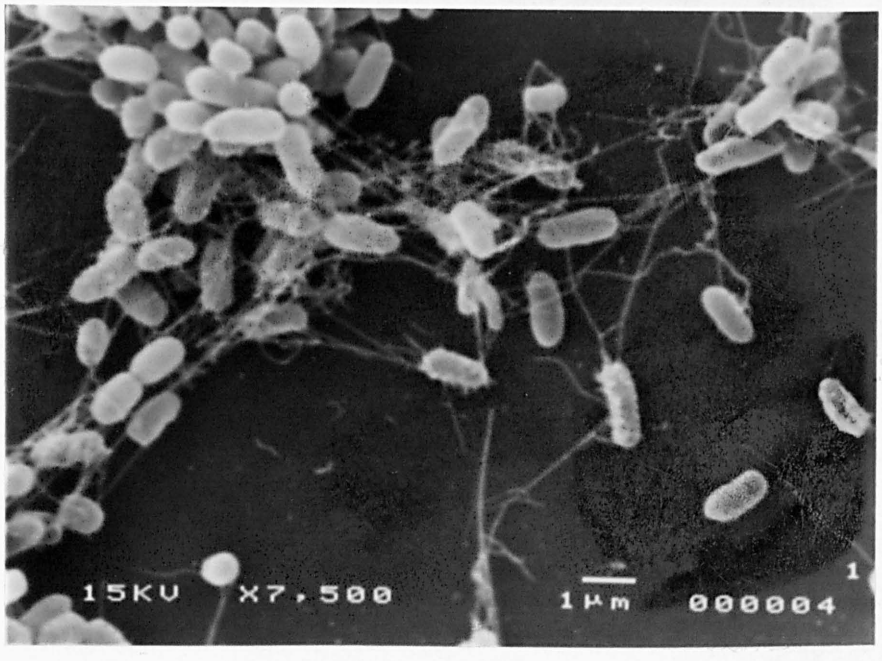
Figure 4.22

Scanning electron micrographs showing mixed culture biofilm development (Nitrogen limited)

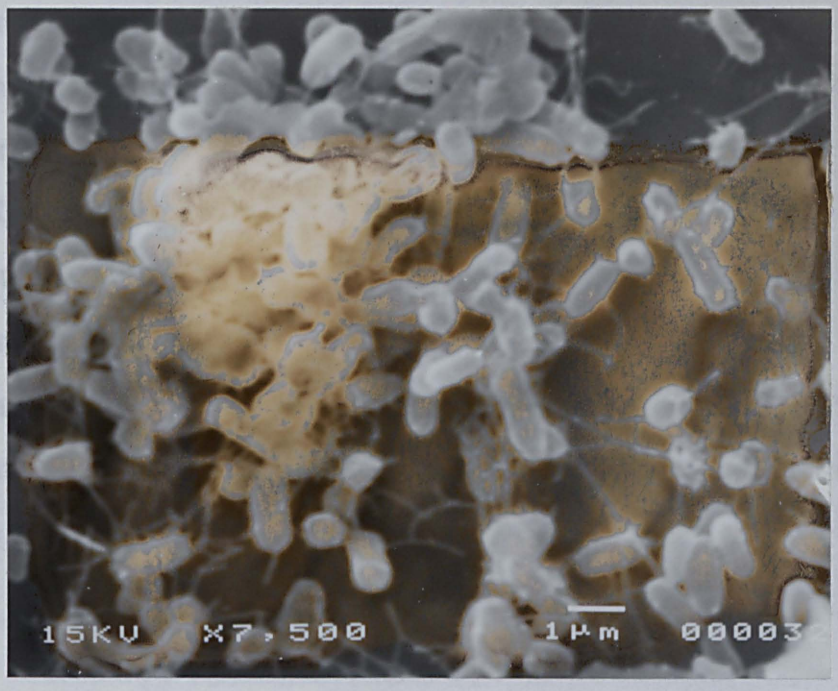
A. 2 hour



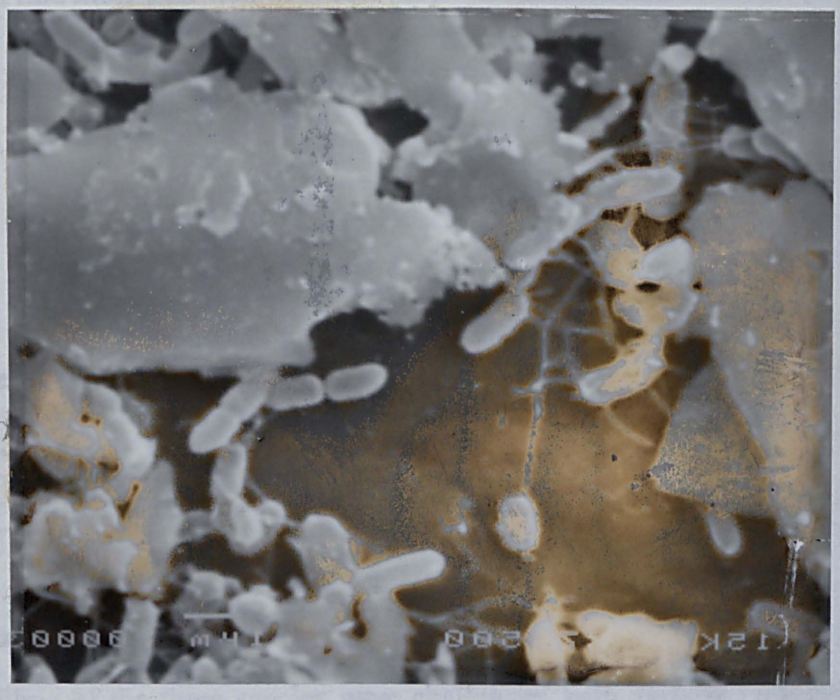
B. 4 hour



C. 8 hour



D. 24 hour



4.6 Stability of an established mixed culture to the colonization by *Escherichia coli* O157 H7

4.6.1 Stability of the liquid phase

A carbon limited chemostat at 0.217 hr^{-1} was challenged with an inoculum of *E.coli* O157 H7, and the persistence of *E.coli* in the liquid phase monitored. Table 4.3 shows that *E.coli* was rapidly washed out of the system to below detectable levels. Under these conditions *S.liquefaciens* was the dominant organism and the results show that *E.coli* was unable to out-compete the resident flora or that some inhibitory compound prevented growth. The inoculum had been previously grown in the same media as that in the chemostat but the organism was unable to become established in the system. The fact that *E.coli* was lost from the system at a faster rate that due to wash out suggests that the resident flora had a biocidal effect on the *E.coli.*

Table 4.3

Stability of the mixed culture chemostat to the presence of *E.coli* O157.

Time after addition of *E.coli* (hr)	Concentration of *E.coli* (cells ml^{-1})
0	$5.55x10^4$
1	$2.0x10^2$
4	<10
6	<10

E.coli was added to the mixed culture carbon limited chemostat (dilution rate 0.217 h^{-1})at steady state, the numbers of *E.coli* were determined by plating onto Eosin Methylene blue agar. The levels of the three species in the steady state population are shown in Fig 4.15.

4.6.2 Stability of a 24 hr tri-species biofilm

Biofilms of the three species were challenged with varying concentrations of *E.coli* O157. Table 4.4 shows that at higher concentrations, *E.coli* was found on the surface in

relatively low numbers, but was not detected on the surface when the inoculum was 10^7 cells ml^{-1}. The concentration of cells on the surface was $4.51x10^7$ cells cm^{-2} therefore a concentration of 10^4 *E.coli* cm^{-2} is a very low proportion of the surface population. The low attachment of *E.coli* may be due to the inhibition of its attachment by the other organisms by steric interference, or alternatively *E.coli* O157 may have poor attachment abilities. However, monoculture controls with *E.coli* alone showed that the organism was able to reach 10^6 cells cm^{-2} under similar conditions.

Table 4.4

Stability of a 24 hour trispecies biofilm to the presence of *E.coli* O157

Concentration of *E.coli* added to the biofilm (cells ml^{-1})	**Attachment ratio ($x10^{-3}$) of *E.coli* detected by antibody labelling**
$2.0x10^7$	None detected
$2.0x10^8$	0.116
$1.0x10^9$	0.390

Mixed species biofilms were grown on the stainless steel discs, triplicate biofilms were challanged by immersion in *E.coli* suspensions at the above concentrations for one hour. *E.coli* that had attached to the surfaces was detected by indirect immunofluoresence using polyclonal antibody and the attachment ratios calculated by dividing the concentration of cells on the surface by that in the liquid phase.

Various workers have examined the attachment interactions between different strains. Cowan *et al.* (1991) found that the colonization of *A.hydrophila* increased in the presence of *P.fluorescens.* Banks and Bryers (1991) found that the establishment of a depositing organism in a biofilm composed of another species was a function of relative growth rates. They found that a *Hyphomicrobium* sp did not become successfully established in

significantly numbers in the *P.putida* biofilm. However, *P.putida* became the dominant organism in the biofilm between 24-48 hr which was originally a *Hyphomicrobium* biofilm. As the organisms in the liquid phase are at steady state they have optimized their growth to those conditions, therefore *E.coli* was disadvantaged and could not out-compete the other organisms to establish itself.

In the biofilm challenge experiment *E.coli* was resuspended in PBS and added to 24 hr biofilms in a static attachment assay for 1 hr. Consequently *E.coli* would have had a reduced growth rate, whilst the organisms of the surface were surrounded by extra-cellular products such as exopolysaccharides which may serve as nutrient reserves.

McEldowney and Fletcher (1987) found that the presence of one species on the surface influenced the attachment of the second organism in various ways. For example increased adhesion of *Acinetobacter calcoaceticus* was found to surfaces pre-treated with a coryneform species, and the adhesion of a *Staphylococcus* species to a surface with an attached population of *A.calcoaceticus* was similarly affected. However, inhibition of attachment and no effect on attachment were also observed. It is possible that the first organism covers the majority of the attachment sites on the surface so that attachment of the second species is due to cell-cell interactions. However, the formation of confluent monolayers are rarely observed and therefore the effects on attachment may be through changes in the surface micro-environment produced by the metabolism of the organisms on the surface that may promote or inhibit attachment.

Relatively high levels of non-spoilage and non-pathogenic organisms may be tolerated in the food processing environment but the presence of pathogens obviously cannot. This work suggests that a resident flora of 'harmless' environmental organisms may reduce the colonization of the surface by a pathogen such as *E.coli,* analogously to the commensal flora of the gut. However *E.coli* was found on the surface in low numbers and the presence of the biofilm consortium may protect the organism from disinfectant action and therefore remain as a source of contamination.

4.7 Comparison of the activity of mixed culture attached and free living populations

Bacterial activity measurements reflect the numbers of metabolically active bacteria in a sample. The synthesis of various cell components is generally measured by following the incorporation of a radio labelled substrate. From the rates calculated, an estimate of the total bacterial activity of a sample is obtained. The activity of a sample does not indicate bacterial numbers, but when used in conjunction with the direct counting techniques, the physiological state of a population can be determined.

The physiological activities of attached and chemostat populations were compared by measuring glucose respiration and tritiated thymidine incorporation. Thymidine incorporation reflects DNA synthesis, and because of the requirement of DNA synthesis for cell division this is the best indicator of cell activity. Thymidine incorporation is only useful as a measurement of bacterial production is several assumptions are made (Carman *et al.*, 1988). Incorporation of the label must be linear with time; radioactivity of the DNA must result from direct incorporation of the thymidine and not from recycling of degradation products if thymidine catabolism; thymidine should not be incorporated into cellular macromolecules. Jeffrey and Paul (1986a) examined the specificity of thymidine labelling as under certain conditions thymidine may be incorporated into protein. They found that at the thymidine concentration used (1 μM) no tritium was incorporated into the protein fraction irrespective of nutrient levels. The possibility that high concentrations of thymidine may result in inhibition of other enzymic pathways preventing label incorporation into protein was also examined, and these workers found that no label was incorporated into protein at 5 nM thymidine. The concentration of thymidine used in this study was 0.1 μM and therefore was in the range where incorporation into protein does not occur.

The rate limiting step for 3H thymidine incorporation into DNA is often the DNA polymerase reaction (Jeffrey and Paul, 1986a). Low extracellular levels of thymidine result in high intracellular levels of dTTP, and the thymidine kinase reaction becomes the rate limiting step. However high levels of thymidine (as used by Jeffrey and Paul (1986 a and b) and in this study) reverse the inhibition of thymidine kinase. Pollard and Moriarty (1984)

demonstrated that isotope dilution was insignificant at thymidine concentrations greater than 35 nM. Therefore, thymidine studies at higher concentrations are a measurement of incorporation rates and any differences observed are not due to thymidine transport kinetics.

The reports in the literature as to the effect of attachment on bacterial activity are contradictory. Some workers have reported increased activity in free cells (Gordon *et al,* 1983) whilst others have reported greater activity in attached cells (Bright and Fletcher, 1983 ; Ellwood *et al.*, 1982 ; Jeffrey and Paul, 1986a ; Iriberri *et al.*, 1990 ; Fletcher, 1986 ; Paerl and Merkel, 1982 ; Unanue *et al.*, 1992). Van Loosdrecht *et al.* (1990) review the effects of attachment on activity reported by various workers.

Table 4.5

Comparison of the activity of attached and liquid phase cells under different nutrient limitations and at different growth rates.

Nutrient limitation	Dilution rate (h^{-1})	Attached (A) or liquid (L) cells	Thymidine incorp. (mole $cell^{-1}h^{-1}$ x10 $^{-20}$)	Glucose resp. (mole $cell^{-1}hr^{-1}$ x10 $^{-18}$)
Nitrogen	0.133	A	226 (± 174)	176 (± 112)
		L	1.06 (± 0.80)	0.504 (± 0.1)
	0.217	A	430.0 (± 172)	302.0 (± 77.0)
		L	3.33 (± 1.09)	10.6 (± 2.10)
Carbon	0.133	A	55.5 (± 11.3)	499.9 (± 141.8)
		L	1.33 (± 1.09)	1.34 (± 2.01)
	0.217	A	65.2 (± 20.1)	679 (± 104)
		L	2.38 (± 1.08)	2.78 (± 0.60)

The activity of 24 hr tri-species biofilm was compared to that of the mixed culture chemostat cells. Figures are means of five replicate samples with standard deviations in brackets.

Table 4.5 shows the activity of attached and free cells as measured by glucose respiration and thymidine incorporation, under carbon and nitrogen limitation and at different dilution rates. The activity experiments compared the activity of 24 hr mixed culture biofilms to a similar concentration of liquid phase cells. Van Loosdrecht *et al.* (1990) reviewed the influence of attachment on microbial activity and suggested that some of the increases in activity observed for attached populations in the literature were in fact due to poor experimental design. They concluded that in certain experiments the number of free cells in the reaction flasks were significantly higher than the number of attached cells, and therefore the free cells may have been subjected to substrate limitation. The experimental procedure used in this study was therefore designed so that this would not be a factor. The volume of chemostat culture added to the reaction flasks was adjusted so that the numbers of cells per flask was similar for both free and attached populations. The biofilm population was rinsed in fresh media (either carbon or nitrogen limited), whilst the chemostat cells were harvested, washed and resuspended to an appropriate concentration.

The results show that attached cells were more active than the liquid phase cells under the conditions tested. Under nitrogen limitation, thymidine incorporation by the attached cells was approximately 100-200 times higher than that observed for liquid phase cells. A similar trend was observed for glucose respiration rates with the activity of the attached population being significantly higher. Both thymidine incorporation and glucose respiration showed an increase in rate with increases in dilution rate for both attached and liquid populations. As increasing dilution rate of a chemostat system increases the growth rate this observation would be expected. However, the attached population appeared to be less affected by the increase in dilution rate than the free cells, as the thymidine incorporation rates of attached cells doubled but was three times higher in free cells at the higher dilution rate. This trend was more obvious in the glucose respiration rates. The attached cell rate doubled as dilution rate increased from 0.133 to 0.217 hr^{-1}, however, the free cells showed a twenty fold increase in respiration.

As already mentioned, the attached population in a chemostat is not subject to the same growth rate control as the liquid phase population. These results suggest that changing the growth rate does have an effect on the attached population but it is not as marked as the effect on liquid phase cells. Similar trends were observed under carbon limitation.

Thymidine incorporation was generally higher in nitrogen limited cells than carbon limited. The reason for this is unclear, perhaps the extent of the nitrogen limitation was not as high as the carbon limitation.

Glucose respiration was generally higher in the carbon limited cells. In these experiments the attached cells were prepared by rinsing the biofilms with fresh carbon limited media and then covering with 2 ml of the same media. The level of carbon was 5 mM, however ^{14}C glucose and 10 mM glucose was added in the assay. Consequently the extent of the limitation was reduced, and this may explain the higher respiration rates observed in the carbon limited cultures. Obviously the nitrogen limited cells were limited by nitrogen and further additions of carbon would not affect the glucose respiration rate.

The activity of the attached populations was higher under both carbon and nitrogen limitation and at both 0.133 hr^{-1} and 0.217 hr^{-1}. Other workers have also observed increased activity in attached populations. Jeffrey and Paul (1986a) examined the activity of attached and free cells of a marine Vibrio sp by measuring thymidine incorporation, p-iodonitrotetrazolium reduction and ATP/DNA ratios. They found that in the presence of exogenous nutrients the activity of the free living cells was greater than that of the attached cells. However, in the absence of peptone-yeast extract the activity was greater in attached cells. It appeared that attached cells were less sensitive to changes in the nutrient concentration than the free population. This observation may tie in with the responses to increases in dilution rate described here as the attached population was affected to a smaller extent.

In the absence of nutrients, Jeffrey and Paul (1986a) found that the thymidine incorporation rate of attached cells was $3.23 \pm 1.26 \times 10^{-20}$ mol $cell^{-1}hr^{-1}$. In similar studies they found attached cells had incorporation rates between $1.01\text{-}10.71 \times 10^{-20}$ mol $cell^{-1}hr^{-1}$, whilst unattached cells incorporated $1.13\text{-}9.53 \times 10^{-20}$ mol $cell^{-1}hr^{-1}$ (Jeffrey and Paul,

1986b). Comparison of these results with the data presented here shows that larger differences were observed between attached and free cells, however the free cell incorporation rates were similar.

Another factor that may play a role in the differences in activity of the two populations is the relative concentrations of the three species in the attached and liquid phases. The chemostat studies showed that *S.liquefaciens* was the dominant organism in the liquid phase, and although the other two organisms were present on the surface in relatively higher numbers than the liquid phase, *S.liquefaciens* was also the most dominant organism on the surface. Consequently the slight changes in the relative concentrations of the three organisms will only have a negligible effect on net activity.

Unanue *et al.* (1992) used incorporation of ^{3}H thymidine and ^{14}C glucose to examine the activity of attached and free living organisms in coastal waters. The attached bacteria were found to be more active than the free living population on a per cell basis, with the attached cells incorporating five times more glucose and twice as much thymidine, although the two populations had similar specific growth rates. The reason for elevated incorporation by the attached population may be because these cells require higher levels of glucose to synthezise extracellular polysaccharides to aid attachment (Kirchmann, 1983). Various other theories have been developed to explain the effect of the attached mode of growth on cell activity. Ellwood *et al.* (1982) suggested that surface associated cells were more active owing to a localized higher concentration of exuded protons. The protons may be passed back into the cell generating ATP and increasing cell activity. Kjelleberg and Dahlback (1984) supported this theory as they reported high ATP levels in starved cells attached to glass. Jeffrey and Paul (1986a) suggested that increased activity observed in attached cells may be due to the micro-environment which may be enhanced by the boundary layer conditions, the presence of EPS and molecular and nutrient adsorption.

Nutrients are thought to adsorb to surfaces in sea water and attached bacteria have been shown to have an ecological advantage in being able to scavenge the adsorbed nutrients (Kefford *et al.*,1982). Consequently higher activities for attached cells in low nutrient environments may be the result of greater availability of scarce nutrients at the surface.

Several workers have reported increased bacterial activity on surfaces in low nutrient environments (Kjelleberg and Dahlback, 1984). However, only a small part (<0.1%) of the bacterial cell surface may be in direct contact with the substratum (Van Loosdrecht *et al.*, 1990).

4.8 Conclusions

The attachment characteristics of *S.liquefaciens, P.fragi* and *S.cohnii* have been examined at different temperatures and pHs, and under anaerobic and nutrient limited conditions. Attachment was shown to be maximal in cells grown at the lowest pH value, and in cells grown at the highest growth temperature examined. The latter suggested that physicochemical adsorption was involved as attachment increased with temperature.

Both the growth media and the phase of growth influenced attachment with the complex media and the late exponential phase reulting in the highest level of attachment of *S.liquefaciens.*

Anaerobiosis was shown to result in a significant reduction in the attachment of *S.liquefaciens,* and in contrast a marked increase in the attachment of *S.cohnii* was observed.

A tri-species mixed culture biofilm was developed using a chemostat system. A stable liquid populaion of the three species was achieved for a defined period. *S.cohnii* was shown to have the highest attachment ratio, and therefore this organism was present in relatively higher numbers on the surface than in the liquid phase. *S.liquefaciens* had the lowest attachment ratio i.e. was present in relatively lower numbers compared to its liquid phase concentration.

Scanning electron micrographs showed that biofilm development under the conditions used in this study did not result in a thick multilayer of cells, but a collection of microcolonies several cells deep with the stainless steel surface visible in places. Extracellular polymer was also observed around the cells, and between the cells and the surface.

The attached population was shown to have a greater activity than the liquid phase population as measured by thymidine incorporation and glucose respiration.

CHAPTER FIVE

5. EFFECT OF GROWTH CONDITIONS ON THE OUTER MEMBRANE AND ATTACHMENT

5.1 Introduction

The results in chapter four demonstrated that bacterial attachment to stainless steel varied with growth conditions. Since, the cell envelope is the primary structure through which a bacterium interacts with its environment its composition and structure and therefore its surface associated properties must reflect the nature of the growth environment (Ellwood and Tempest, 1972).

The outer membrane of the Gram negative cell consists of lipopolysaccharides (LPS), outer membrane proteins (OMP), lipoproteins and phospholipids, however, the outer surface of the outer membrane consists of only LPS and OMP. Although LPS molecules are tightly packed on the outer surface of the outer membrane, about 3.5 million molecules per cell surface area of about 6 μm^2 in *E.coli* (Nikaido and Vaara, 1987), the O-antigenic polysaccharide chains are not the only molecules visible at the cell surface. Proteins make up approximately half the weight of the outer membrane and at least 20 of these proteins have been shown to be surface antigens (Hancock, 1991). Consequently, it was decided to investigate the effects of various growth conditions on outer membrane protein and LPS profiles as these are the components most likely to interact with the environment and therefore play a role in attachment.

A variety of growth conditions have been shown to influence OMP and LPS profiles including temperature (Yamamori and Yura, 1980; Lugtenberg and Van Alphen, 1983); nutrient limitation (Lugtenberg and Van Alphen, 1983; Griffiths *et al.*, 1983; Anwar *et al.*, 1991); osmolarity (Lugtenberg and Van Alphen, 1983); pH (Knox *et al.*, 1985); growth rate (Anwar *et al.*, 1991); *in vivo* growth (Brown *et al.*, 1984a; Sutherland *et al.*, 1990); anaerobiosis (Davies *et al.*, 1992) and growth phase (Davies *et al.*, 1991). This chapter describes the effects of various growth conditions on OMP and LPS profiles and relates the observed changes to differences in attachment found with these growth conditions.

5.2 Aims

The objective was to examine the effects of a range of growth conditions including nutrient limitation, anaerobiosis, pH and temperature on the OMP and LPS profiles of *S.liquefaciens* and *P.fragi*. Although Gram positive bacteria such as *S.cohnii* do possess proteinaceous outer layers the methods in used in this chapter are not appropriate for their isolation and therefore the results presented concentrate on the two Gram negative bacteria. The results are discussed in relation to the attachment data described in chapter four. The effect of trypsin on the outer membrane was also investigated as this was found to affect the attachment to stainless steel. Finally, OMP profiles of attached cells were compared to liquid phase cell profiles.

5.3 Effect of nutrient limitation on the outer membrane

5.3.1 OMP profiles

Figure 5.1 shows the OMPs detected by silver staining of SDS polyacrylamide gels. Several major protein bands were visible in both organisms with numerous faint minor protein bands also present. Figure 5.1(a) shows that *P.fragi* expressed an additional 57kDa protein (indicated by the arrow) under carbon limitation that was not detected under the other growth conditions. Similarly, *S.liquefaciens* produced an additional protein (14kDa) under carbon limitation (indicated by the arrow in Figure 5.1(b)). Nitrogen limitation appears to result in the synthesis or increased expression of a 67 KDa protein. These additional proteins may be involved in uptake systems.

The minor outer membrane proteins have been reported to be more immunogenic than the major bands and are therefore present as the dominant bands in Western blot analysis (Davies *et al.*, 1990). Consequently Western blot analysis was performed using the polyclonal antibodies previously raised for immunofluorescent labelling in order to obtain additional information about the effect of nutrient limitation on OMP expression. Figure 5.2 shows that the minor OMP are in fact more immunogenic than the major proteins as the antibodies detected significantly more protein bands.

Figure 5.1

SDS-PAGE showing the effect of nutrient limitations on the OMP profiles of (a) *P.fragi* and (b) *S.liquefaciens*

OMP preparations were added to the tracks as indicated (5μg protein per track)

Key to tracks

A: *P.fragi* - carbon limited

B: *P.fragi* - nitrogen limited

C: *P.fragi* - carbon and nitrogen excess

D: *S.liquefaciens* - carbon limited

E: *S.liquefaciens* - nitrogen limited

F: *S.liquefaciens* - carbon and nitrogen excess

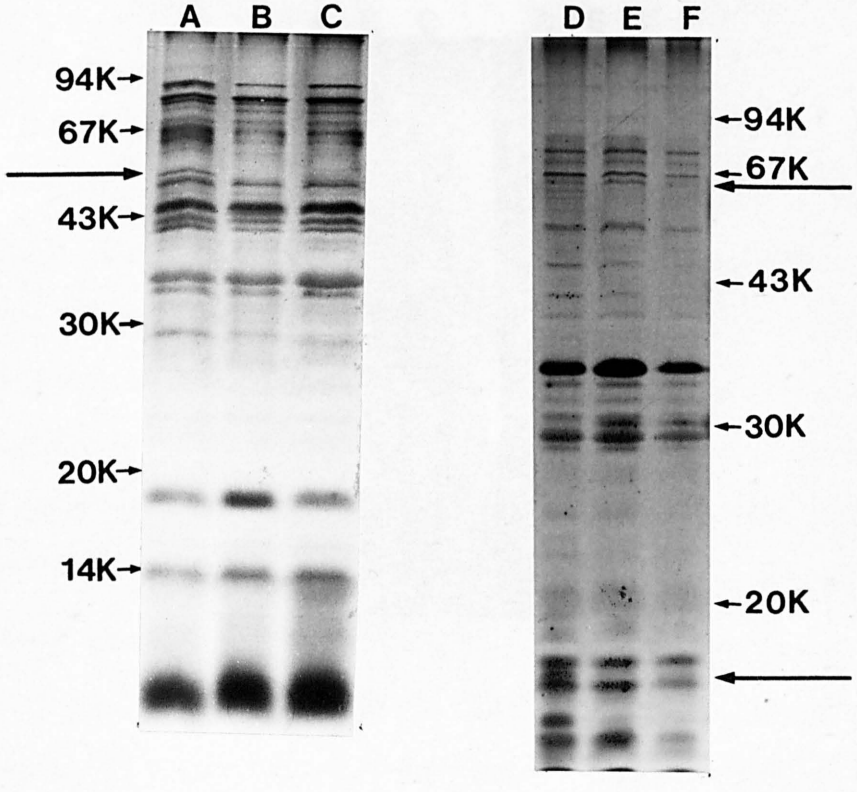


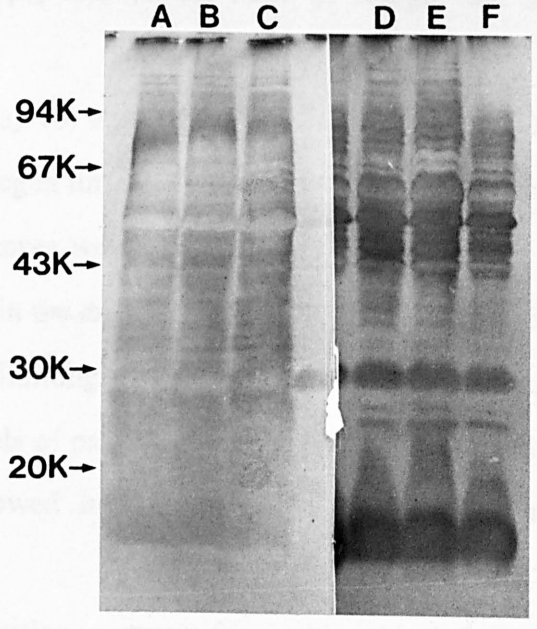
Figure 5.2

Western blot of nutrient limited OMP profiles using polyclonal antisera

Proteins were separated on a 15% linear SDS-polyacrylamide gel (20µg protein per track) and transferred to nitrocellulose. The antibody complexes were detected as described in the materials and methods (2.32.2).

Key to tracks

A: *P.fragi* - carbon limited
B: *P.fragi* - nitrogen limited
C: *P.fragi* - carbon and nitrogen excess
D: *S.liquefaciens* - carbon limited
E: *S.liquefaciens* - nitrogen limited
F: *S.liquefaciens* - carbon and nitrogen excess



Although there appeared to be no detectable differences in the *P.fragi* tracks, *S.liquefaciens* exhibited reduced expression of the proteins in the areas indicated by the arrows when grown in excess carbon and nitrogen media which would suggest that carbon or nitrogen limitation results in increased expression of specific proteins.

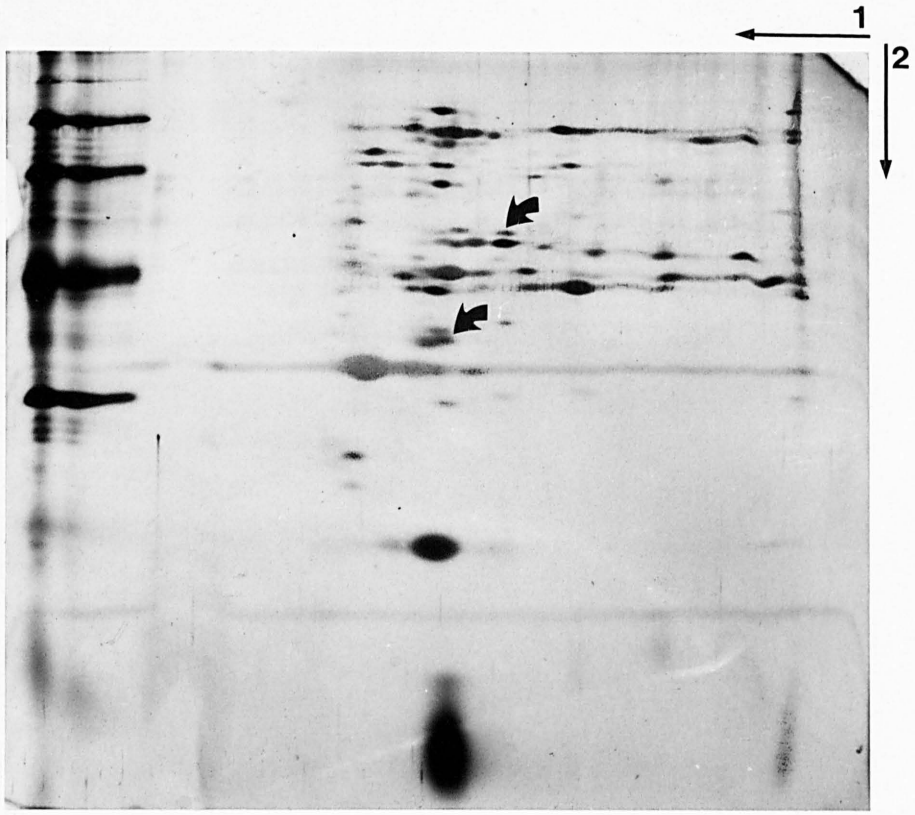
It is interesting to note that the *P.fragi* antisera used on the *S.liquefaciens* track reacted with two major protein bands, although there was no cross reaction detected in immunofluorescent labelling experiments *P.fragi* and *S.liquefaciens* therefore possess antigenically similar OMP.

Separating polypeptides on the basis of molecular weights in one dimensional gels means that two or more polypeptides may comigrate as a single band. The comigration of polypeptides may mask the true complexity of the proteins present and it can also mask any quantitative changes in the components present in any one band. For these reasons two dimensional electrophoresis was used with isoelectric focusing in the first dimension, so separating the polypeptides on the basis of charge, and molecular weight in the second dimension.

Figure 5.3 (a), (b) and (c) show the silver stained 2D gels of *P.fragi* OMP grown in carbon limited, nitrogen limited and carbon and nitrogen excess media respectively. Certain reproducible differences were observed and are indicated by the arrows. Generally limiting conditions resulted in the expression of additional proteins. Similar trends were observed for *S.liquefaciens* with limiting conditions resulting in the production of new proteins or in some cases increased levels of particular proteins. These observations confirmed the Western blot analysis which showed increased levels of minor protein bands with limiting growth conditions.

These observations compare favourably with the effects of nutrient limitation reported in the literature. Brown and Williams (1985) found that phosphate, sulphur and iron limitation resulted in the induction of outer membrane proteins in *E.coli.* These workers also reported that phosphate, carbon, sulphate and potassium limitation induced specific outer membrane proteins in *Klebsiella pneumoniae.* Since changes in the cell wall composition of Gram negative bacteria (in response to specific nutrients) are mainly due to porin synthesis

(a)



(b)

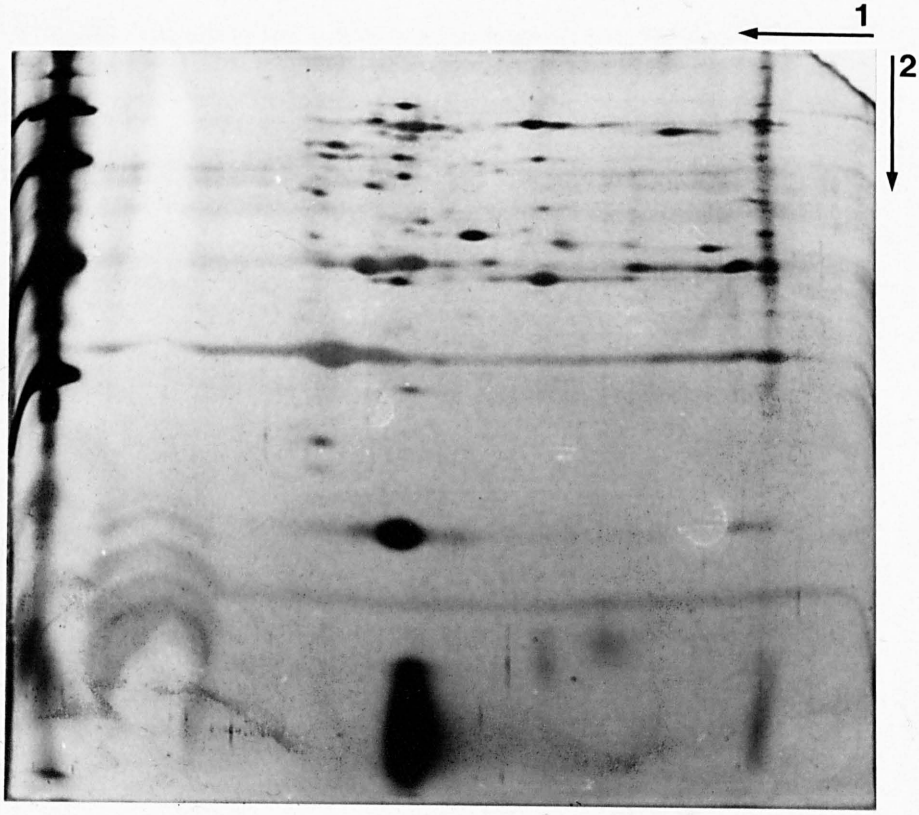
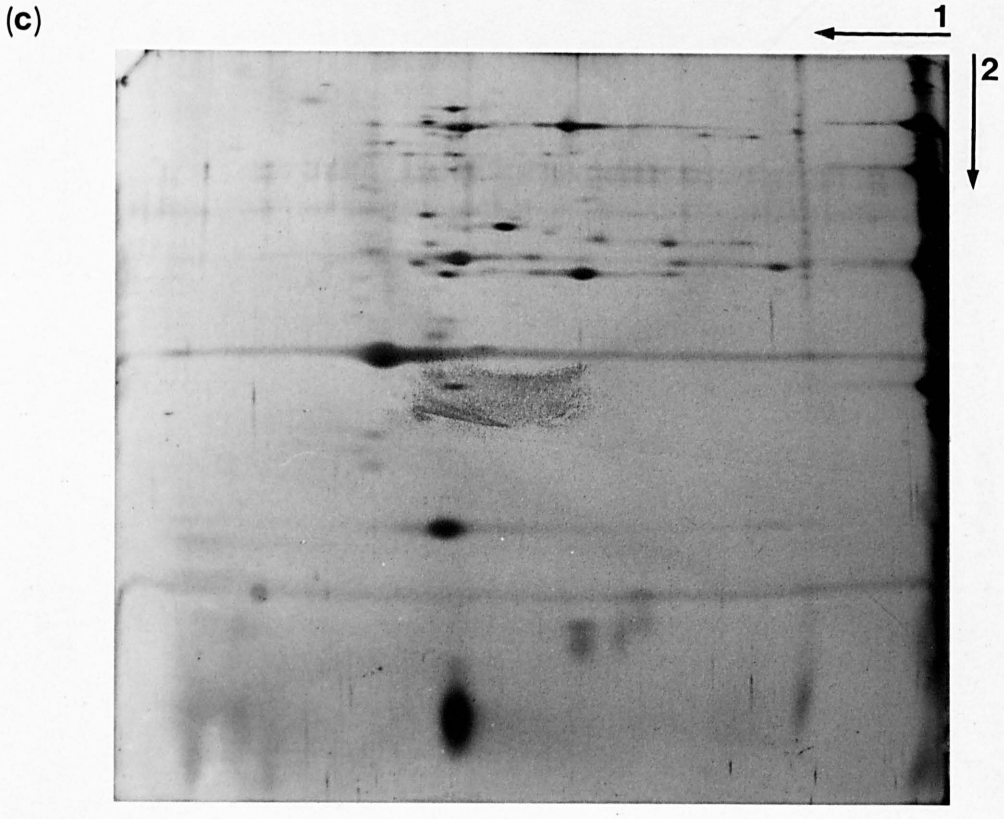


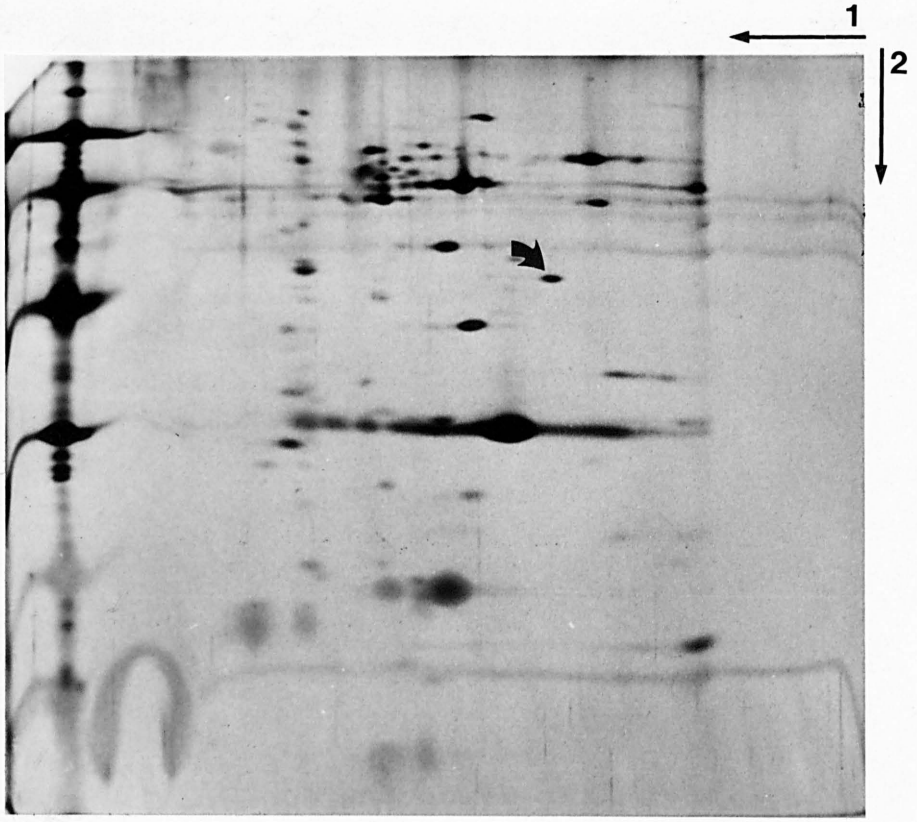
Figure 5.3

Two dimensional electrophoresis of *P.fragi* OMPs isolated from cells grown under (a) carbon limitation, (b) nitrogen limitation or (c) carbon and nitrogen excess.

Samples containing 10 μg protein were loaded onto the isoelectric focusing gels. Standard protein markers were run in the second dimension (12% linear SDS-polyacrylamide gel).



(a)



(b)

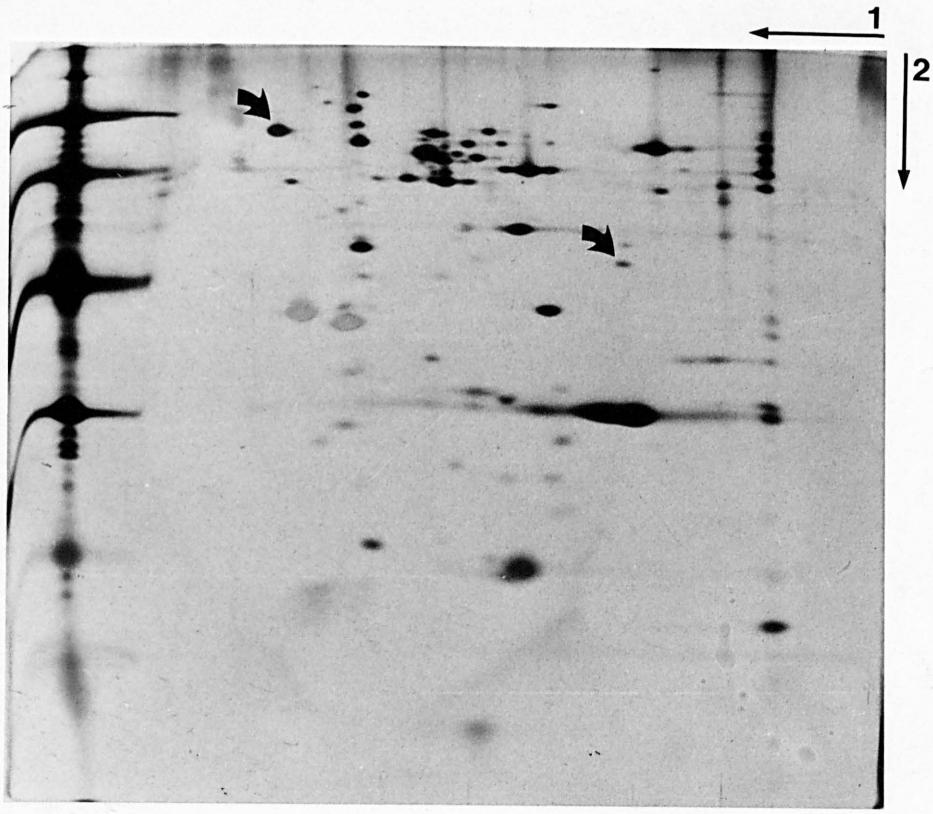
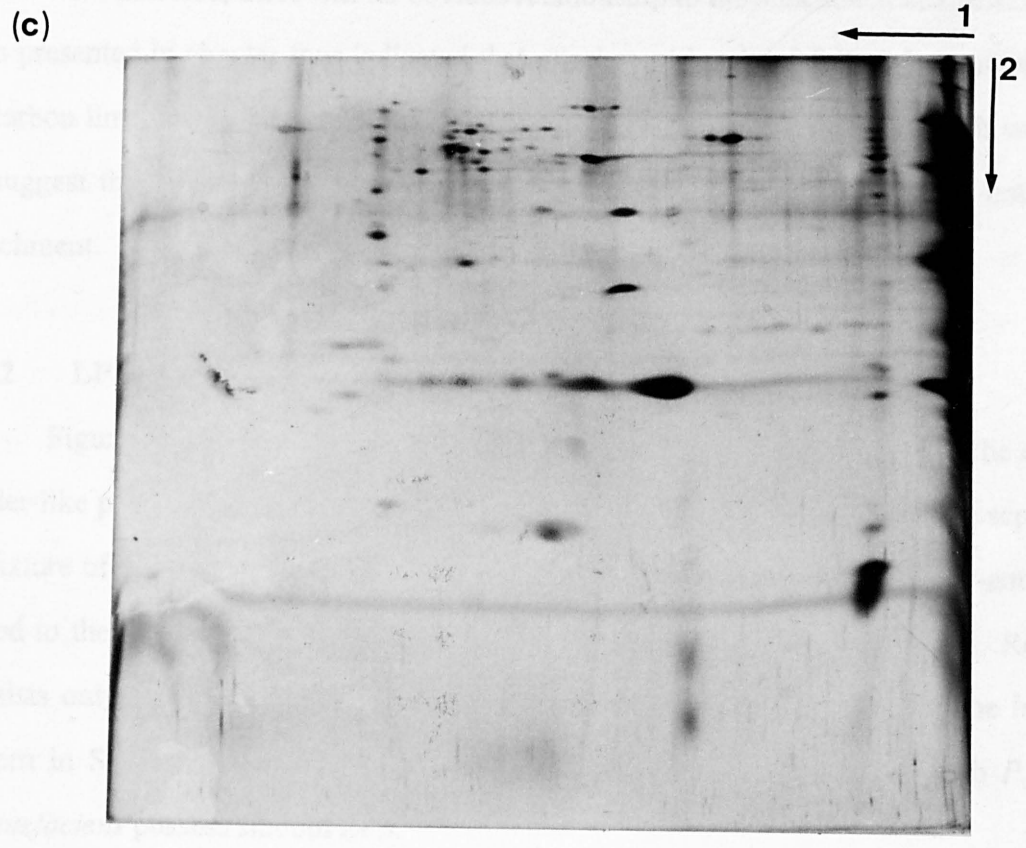


Figure 5.4

Two dimensional electrophoresis of *S.liquefaciens* OMPs isolated from cells grown under (a) carbon limitation, (b) nitrogen limitation or (c) carbon and nitrogen excess.

Samples containing 10 μg protein were loaded onto the isoelectric focusing gels. Standard protein markers were run in the second dimension (12% linear SDS-polyacrylamide gel).



and the induction of specific uptake mechanisms for the limited nutrient e.g. iron limitation results in the expression of novel OMPs in a number of Gram negative bacteria (Anwar *et al.*, 1991).

Changes in the cell envelope outer proteins (other than integral membrane OMPs) have been implicated in cell adhesion phenomenon. For example, adhesion of pathogenic *E.coli* to the intestine is mediated by non-mannose sensitive pilus adhesins (Sherman *et al.*, 1985). However, Pringle *et al.* (1983) examined the OMP profiles of wild type and attachment deficient mutants of a freshwater bacterium and although small differences in the OMPs were detected, there was no obvious relationship to the attachment characteristics. The data presented in chapter four indicated that attachment levels of *S.liquefaciens* were similar in carbon limited, nitrogen limited and carbon and nitrogen excess media which would seem to suggest that the differences in OMPs observed with nutrient limitation do not influence attachment.

5.3.2 LPS profiles

Figure 5.5 shows the effect of nutrient limitation on LPS expression. The distinctive ladder-like pattern of smooth LPS in SDS-polyacrylamide gels is caused by the separation of a mixture of molecules of increasing molecular mass representing additional O-antigen units added to the basic lipid A core oligosaccharide structure (Davies *et al.*, 1991). Rough LPS consists only of the lipid A core oligosaccharide and does not give rise to the ladder like pattern in SDS-polyacrylamide gels. It can therefore be concluded that both *P.fragi* and *S.liquefaciens* possess smooth LPS.

Figure 5.5 indicates that *P.fragi* produces similar LPS profiles under carbon limitation, nitrogen limitation or carbon and nitrogen excess, although it appears that the levels of LPS were slightly lower in cells grown under carbon limitation. The same volume of proteinase K extracts were loaded per track, however, although the enzyme was added to outer membrane preparations adjusted to the same protein concentrations, increased expression of certain OMP under carbon limitation only would mean that the differences in LPS expression are due to differences in the amount loaded to each track. However, the

reduction in the levels of LPS observed are feasible. The smooth LPS molecule consists of three distinct regions; lipid A, the core oligosaccharide, and the O specific antigen and are tightly packed on the outer surface of the outer membrane. It is possible that when the carbon source is limited (as opposed to carbon and nitrogen excess, or nitrogen limited media with excess carbon) there is a reduction in the concentration of LPS molecules at the surface so that the available carbon can be used for other purposes such as exopolysaccharide synthesis. Indeed Nikaido and Nakae (1980) found that changes in the LPS composition did result in differences in the coverage of the cell surface by the polysaccharide side chains of LPS.

The differences in the LPS profiles of *S.liquefaciens* were more obvious and, as with *P.fragi*, were observed under carbon limitation producing decreased levels of some bands and increased levels of others (as indicated by the arrows).

There is little information in the literature as to the effect of nutrient limitation on LPS profiles although Tsai *et al.* (1983) observed changes in the LPS profile of *Neisseria gonorrhoea* with medium composition. The effect of *in vivo* growth in the peritoneal chamber has been examined, and differences in the LPS profiles have been described for *Pseudomonas aeruginosa* (Kelly *et al.*, 1989) and for *Neisseria gonorrhoea* (Mandrell *et al.*, 1990).

Growth in carbon limited, nitrogen limited and carbon and nitrogen excess media resulted in similar levels of attachment of *S.liquefaciens* to stainless steel, and therefore the differences in the LPS profiles would appear not to influence attachment.

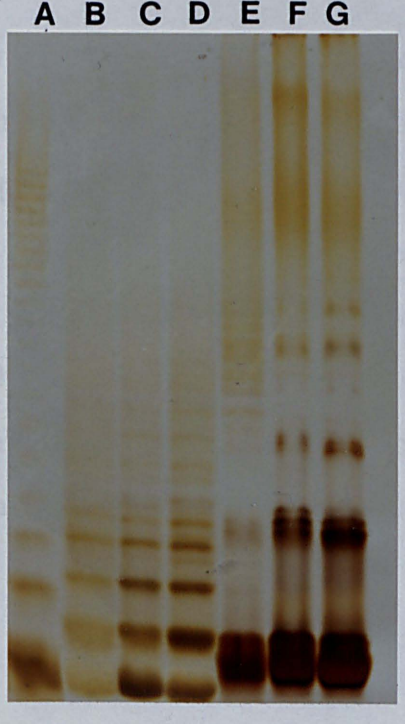
Figure 5.5

Modified SDS-PAGE showing the effect of nutrient limitation on the LPS profiles of *S.liquefaciens* and *P.fragi*

Proteinase K extracts of OMP preparations were prepared by adding the enzyme to preparations with the same concentration of protein. The volumes of the extracts added are shown in the key below. The 15% polyacrylamide gel also contained 4M urea (see materials and methods).

Key to tracks

A: *E.coli* O111
B: *P.fragi* - carbon limited (15µl)
C: *P.fragi* - nitrogen limited (15µl)
D: *P.fragi* - carbon and nitrogen excess (15µl)
E: *S.liquefaciens* - carbon limited (10µl)
F: *S.liquefaciens* - nitrogen limited (10µl)
G: *S.liquefaciens* - carbon and nitrogen excess (10µl)



5.4 Effect of growth temperature

5.4.1 OMP profiles

The effect of growth at 15, 25, 30 and 37°C on OMP expression was examined and several differences were found in *P.fragi* OMP at different temperatures (Figure 5.6). Increased levels of a 28kDa protein were observed in 15°C grown cells and several protein bands 43, 53 and 67kDa appeared or showed increased expression at 25°C (indicated by the arrows). In cells grown at 30°C, a 37kDa protein was detected which was not present at other temperatures.

The OMP profile of *S.liquefaciens* did not show such marked differences with temperature, although a general increase in the levels of the major protein bands was observed.

Temperature is one of the parameters reported to influence OMP profiles (Ellwood and Tempest, 1972; Yamamori and Yura, 1980; Lugtenberg and Van Alphen, 1983) and the results presented confirm that the OMP profiles of *P.fragi* and *S.liquefaciens* vary with temperature.

The data in chapter four showed that the attachment of *S.liquefaciens* and *P.fragi* increased with increasing growth temperatures up to 37°C. Although various differences in OMPs were detected with temperature with particular protein bands induced at specific temperatures, there was no apparent correlation between attachment and OMPs. *S.liquefaciens* showed increased expression of certain OMPs with increases in temperature, and it is possible this related to the increases in attachment observed. However, it is unlikely that these slight differences were responsible for the marked increases in attachment, and the nutrient limitation data did not show any relationship between OMPs and attachment.

Figure 5.6

SDS-PAGE showing the effect of growth temperature on the OMP profiles of *P.fragi* and *S.liquefaciens*

OMPs were prepared from cultures grown to stationary phase in carbon and nitrogen excess medium at 15, 25, 30 and 37°C. Samples containing 7μg protein were loaded to each track

Key to tracks

A: *P.fragi* grown at 15°C
B: *P.fragi* grown at 25°C
C: *P.fragi* grown at 30°C
D: *S.liquefaciens* grown at 15°C
E: *S.liquefaciens* grown at 25°C
F: *S.liquefaciens* grown at 30°C
G: *S.liquefaciens* grown at 37°C

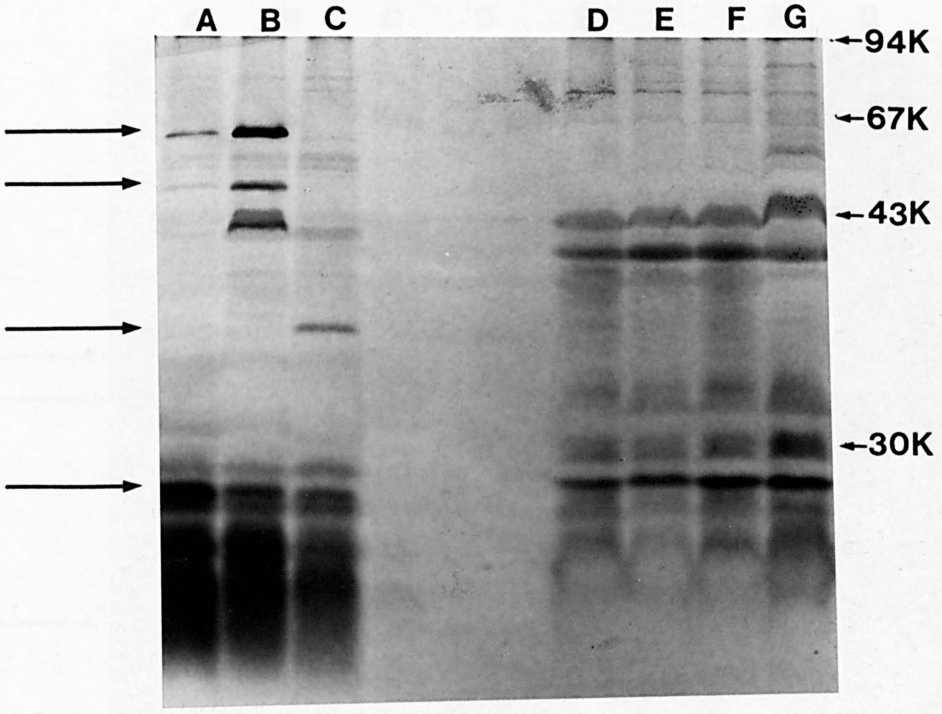


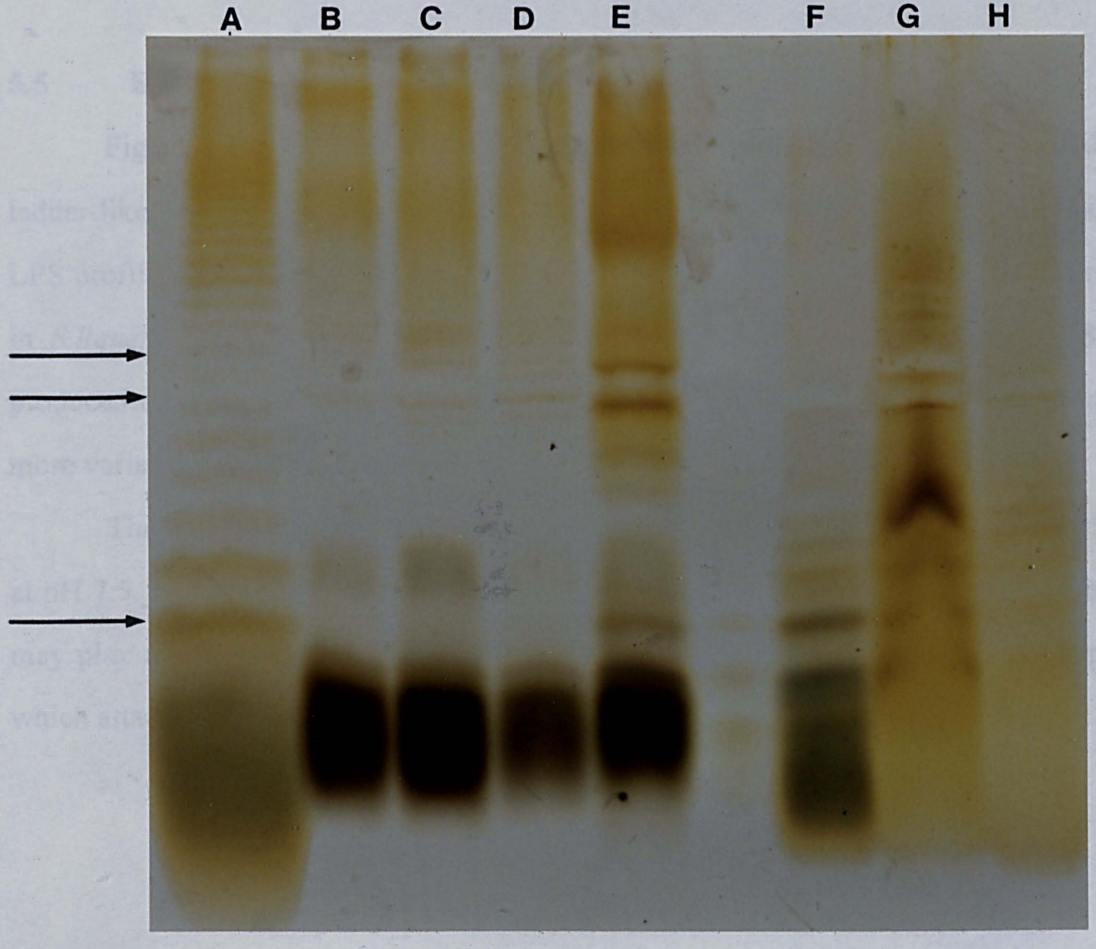
Figure 5.7

Modified SDS-PAGE showing the effect of growth temperature on the LPS profiles of *P.fragi* and *S.liquefaciens*

LPSs were extracted from the OMP preparations described in Figure 5.6 with proteinase K and 6µl added per track.

Key to tracks

A: *E.coli* O111 (20µl)
B: *S.liquefaciens* grown at 15°C
C: *S.liquefaciens* grown at 25°C
D: *S.liquefaciens* grown at 30°C
E: *S.liquefaciens* grown at 37°C
F: *P.fragi* grown at 15°C
G: *P.fragi* grown at 25°C
H: *P.fragi* grown at 30°C



5.4.2 LPS profiles

Figure 5.7 shows the LPS profiles of *P.fragi* and *S.liquefaciens* grown at different temperatures. The marked differences in OMPs of *P.fragi* at 25°C were also noted in the LPS profile at this temperature. Although there were no obvious differences in OMPs of *S.liquefaciens* with temperature, the LPS profile shows variation with temperature with several bands produced or showing increased levels at 37°C (indicated by the arrows).

Attachment was found to be maximal at 37°C in both organisms (chapter four), however, as with the OMP profile of *P.fragi* there appeared to be no correlation with attachment. The differences in the *S.liquefaciens* profile were particularly obvious at 37°C, and therefore LPS may play a role in determining the attachment characteristics of *S.liquefaciens*. This organism produces relatively little extracellular polysaccharide material compared to *P.fragi* (discussed in chapter six) and therefore LPS molecules may be more likely to be 'visible' at the cell surface and influence attachment through contributions to cell surface hydrophobicity and charge (discussed in chapter seven).

5.5 Effect of growth pH

Figure 5.8 shows the effect of growth at different pHs on the LPS profiles. The ladder-like pattern of smooth LPS was particularly obvious in these gels. Differences in the LPS profiles of *P.fragi* with pH were not detected. However, additional bands were present in *S.liquefaciens* grown at pH 6.5 (marked by arrows). Analysis of the LPS profiles produced under different growth conditions indicated that the profile of *S.liquefaciens* was more variable than that of *P.fragi*.

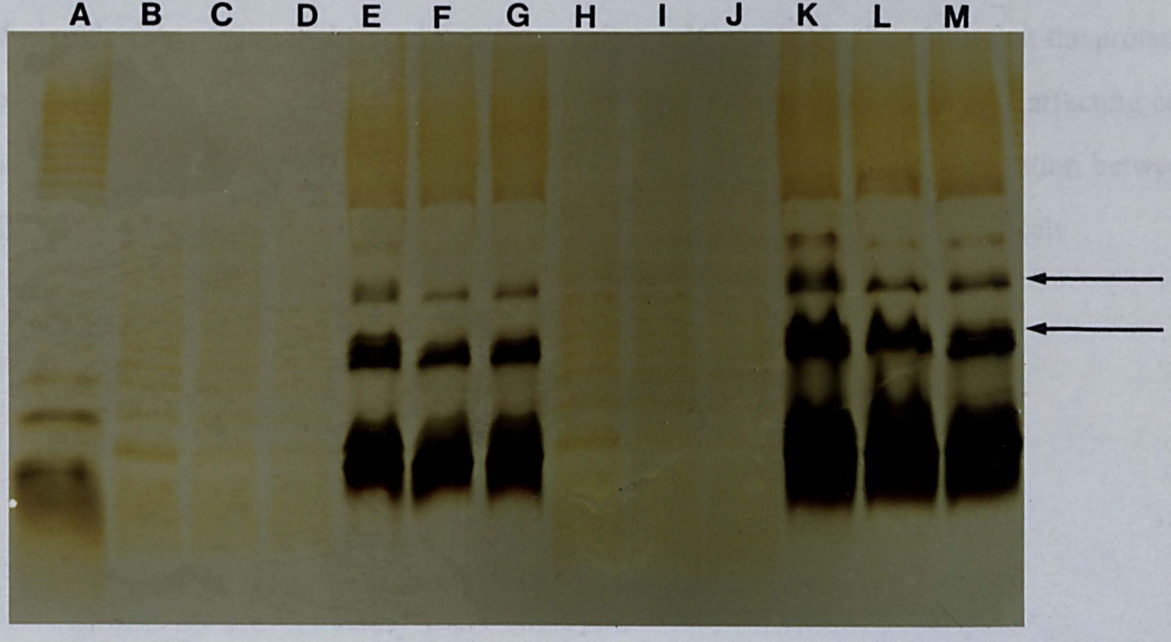
The results in chapter four showed that attachment was higher at pH 6.5 and 8.5 than at pH 7.5. Differences in *S.liquefaciens* LPS were detected at pH 6.5 and therefore the LPS may play a role in attachment, however, no obvious differences were obvious at pH 8.5 at which attachment was equally high.

Figure 5.8

Modified SDS-PAGE showing the effect of growth pH on LPS profiles of *S.liquefaciens* and *P.fragi*

Key to tracks

A: *E.coli* O111 (20µl)
B: *P.fragi* - pH 6.5 (6µl)
C: *P.fragi* - pH 7.5 (6µl)
D: *P.fragi* - pH 8.5 (6µl)
E: *S.liquefaciens* - pH 6.5 (6µl)
F: *S.liquefaciens* - pH 7.5 (6µl)
G: *S.liquefaciens* - pH 8.5 (6µl)
H: *P.fragi* - pH 6.5 (12µl)
I: *P.fragi* - pH 7.5 (12µl)
J: *P.fragi* - pH 8.5 (12µl)
K: *S.liquefaciens* - pH 6.5 (12µl)
L: *S.liquefaciens* - pH 7.5 (12µl)
M: *S.liquefaciens* - pH 8.5 (12µl)



5.6 Effect of growth phase

The effect of growth phase on OMP expression is shown in Figure 5.9. As the same amount of protein was loaded per track, differences in band intensity reflect changes in levels of expression.

Figure 5.9(a) shows that stationary phase *S.liquefaciens* gave increased expression of 16.5, 17.5 and 31 KDa proteins, as well as several high molecular weight minor bands. Two bands at 14 and 30 KDa were found only in exponential phase cells.

Differences in OMP profiles with growth phase were also observed in *P.fragi* (Figure 5.9(b)). Several bands were present only in stationary phase cultures (as indicated by the arrows) with molecular weights of 38, 42, and 51 KDa. The 32 KDa protein was only detected in exponential phase cells.

The effects of growth phase on OMP expression have been examined by various workers (Loeb and Smith, 1980; Kelly and Parker, 1981; Blaser *et al.*, 1983; Davies *et al.*, 1991). Davies *et al.* (1992) found increased expression of certain proteins and decreased levels of others between the exponential and stationary phase and certain proteins were only detected in one or other growth phase.

The attachment experiments showed that attachment was maximal in *S.liquefaciens* during the late exponential to early stationary phase. It is possible therefore, that the proteins produced in the stationary phase may have indirectly influenced attachment by affecting cell surface charge and hydrophobicity (discussed in chapter seven) but as a correlation between OMP and attachment was not observed in the other experiments this seems unlikely.

5.7 Effect of trypsin modification

5.7.1 OMP profiles

Trypsin was used on whole cells of *S.liquefaciens* and *P.fragi* to modify the proteins present on the cell surface in order to determine the relationship between outer membrane proteins and attachment.

The enzyme did not have any effect on the major proteins of *S.liquefaciens* detected by SDS-polyacrylamide gel electrophoresis (Figure 5.10). The additional bands present in the track of OMPs treated with heat inactivated trypsin can be attributed to the protein of the trypsin itself. These data suggests that the proteins detected in the gel are not present at the cell surface and therefore cannot be modified by the enzyme.

As already mentioned, Western blot analysis is a more sensitive method for the analysis of the minor protein bands as they are often more immunogenic. Figure 5.11 shows that trypsin did not produce any detectable differences in the protein profile of *S.liquefaciens*.

Figure 5.10 shows that in contrast to *S.liquefaciens*, the protein profile of *P.fragi* was significantly affected by trypsin in that two major bands were removed by the enzyme (30 and 37 KDa). It is perhaps somewhat surprising that a loss in viability was not detected after treatment with the enzyme considering these major changes. An additional band was detected in the OMP track treated with heat inactivated trypsin, however this may have been a consequence of binding to an OMP retarding its migration. This is confirmed by the Western blot (Figure 5.11) which shows that the additional band detected was in fact a cellular protein.

The attachment studies showed that both active and inactive trypsin reduced the attachment of *S.liquefaciens* (chapter four). As both active and inactive enzyme had similar effects and no differences in the OMP were detected, the effect on attachment must be through binding of the trypsin to the cell surface thus affecting cell surface properties such as charge and hydrophobicity (discussed in chapter seven). The avidity of the binding is demonstrated by the presence of enzyme protein in the *S.liquefaciens* track treated with inactive enzyme. The trypsin did not affect the major OMPs of *S.liquefaciens* suggesting that

these proteins may not be 'visible' at the cell surface. If this is the case then it is unlikely that these proteins play a role in attachment.

The attachment of *P.fragi* after trypsin treatment showed that the enzyme reduced attachment, however inactive trypsin decreased the attachment further. Perhaps the action of the enzyme (removal of two major protein bands) reduced that attachment of the cell, but the adsorption of the inactive trypsin had a larger effect on attachment. Trypsin can obviously adsorb or interact with the cell surface, as demonstrated by its affect on the mobility of certain proteins in the gel.

The susceptibility of the proteins to proteolytic attack suggests that the molecules are located on the external surfaces of the cell wall, however, it cannot be ruled out that the proteins are buried in the wall and that the trypsin acts indirectly by hydrolysing a molecule which is responsible for anchoring these proteins to the cell wall.

5.7.2 LPS profiles

The effect of trypsin on the LPS profiles of *S.liquefaciens* and *P.fragi* was also examined as modification by the enzyme may affect molecules responsible for anchoring the LPS molecules. However, Figure 5.12 shows that there were no marked differences in the LPS profiles of *S.liquefaciens* or *P.fragi* treated with active or inactive enzyme as compared to the untreated control tracks.

Figure 5.9

SDS-PAGE showing the effect of growth phase on OMP expression in (a) *S.liquefaciens* and (b) *P.fragi*

Cells grown in LB broth were harvested at the mid exponential or stationary phase and OMP extracted. Samples were adjusted so that comparable tracks contained the same amount of protein.

Key to tracks

A: *S.liquefaciens* - stationary phase (7µg)

B: *S.liquefaciens* - exponential phase (7µg)

C: *P.fragi* - exponential phase (5µg)

D: *P.fragi* - stationary phase (5µg)

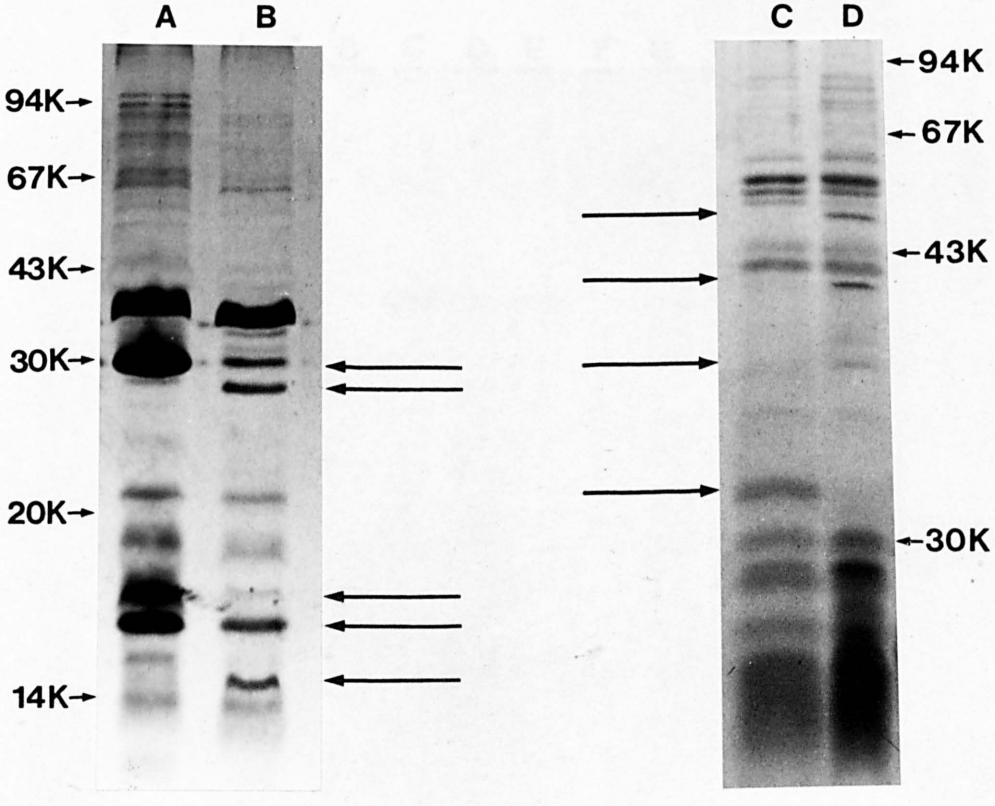


Figure 5.10

SDS-PAGE showing the effect of trypsin on the OMP profiles of *S.liquefaciens* and *P.fragi*

Whole cells were treated with 1%(w/v) trypsin or heat inactivated trypsin for 1 hr at 37°C and the OMP prepared. Samples of active and heat inactivated trypsin are included.

Key to tracks

A & I: Active trypsin

B & J: Inactive trypsin

C: *S.liquefaciens* treated with active trypsin (6µg protein)

D: *S.liquefaciens* treated with inactive trypsin (6µg protein)

E: *S.liquefaciens* untreated control (6µg protein)

F: *P.fragi* treated with active trypsin (4µg protein)

G: *P.fragi* treated with inactive trypsin (4µg protein)

H: *P.fragi* untreated control (4µg protein)

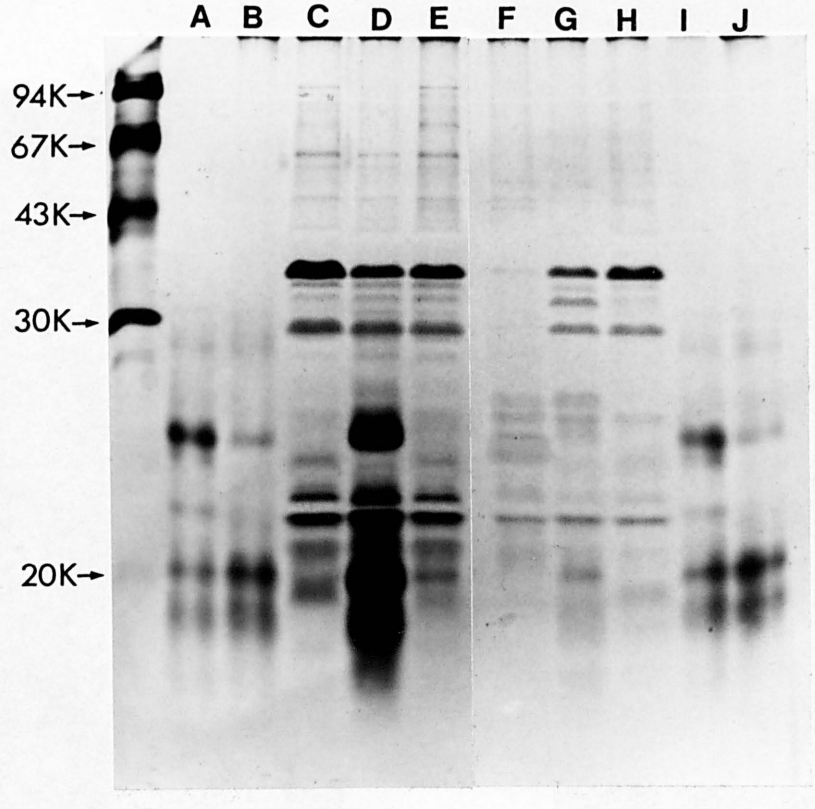


Figure 5.11

Western blot showing the effect of trypsin on the OMP profiles of *P.fragi* and *S.liquefaciens*

Samples were treated as described in Figure 5.10, and 20μg protein loaded per track. After separation on a 15% linear SDS-polyacrylamide gel, proteins were transferred to nitrocellulose and the antibody complexes detected as described in the materials and methods.

Key to tracks

A & I: Active trypsin

B & J: Inactive trypsin

C: *S.liquefaciens* treated with active trypsin

D: *S.liquefaciens* treated with inactive trypsin

E: *S.liquefaciens* untreated control

F: *P.fragi* treated with active trypsin

G: *P.fragi* treated with inactive trypsin

H: *P.fragi* untreated control

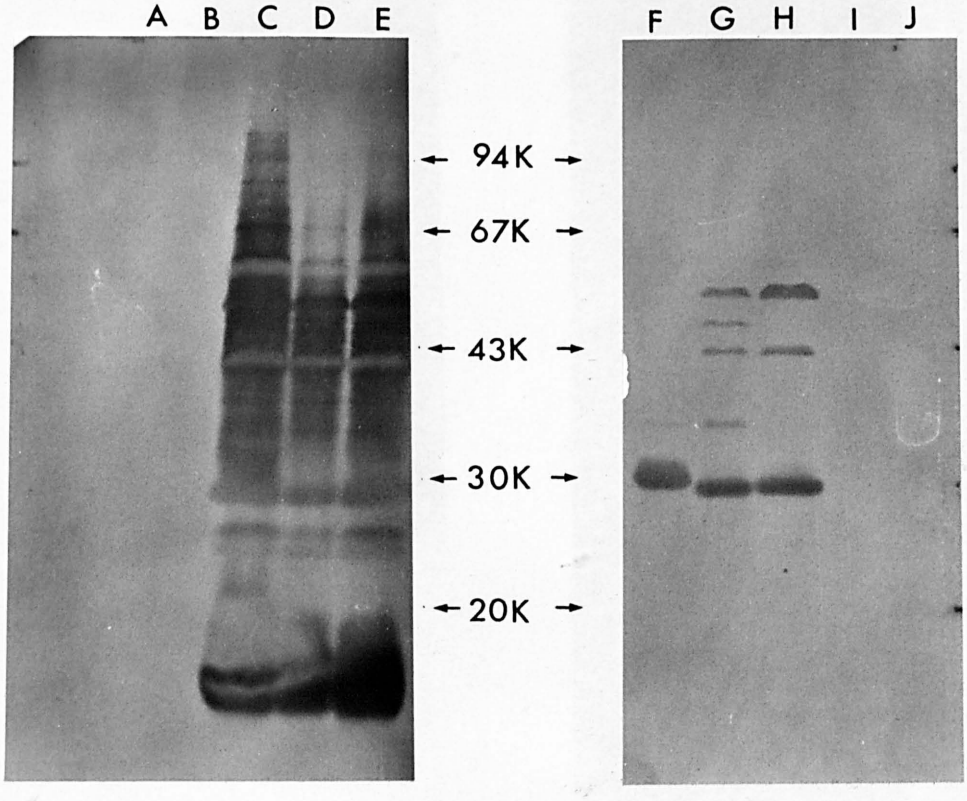


Figure 5.12

Modified SDS-PAGE showing the effect of trypsin on the OMP profiles of *P.fragi* and *S.liquefaciens*

Samples were prepared as described in Figure 5.10. LPS was extracted with proteinase K and 8 µl added per track of all samples. 5µl was used of the *E.coli* O111 control.

Key to tracks

A & E: *E.coli* O111

B: *S.liquefaciens* untreated control

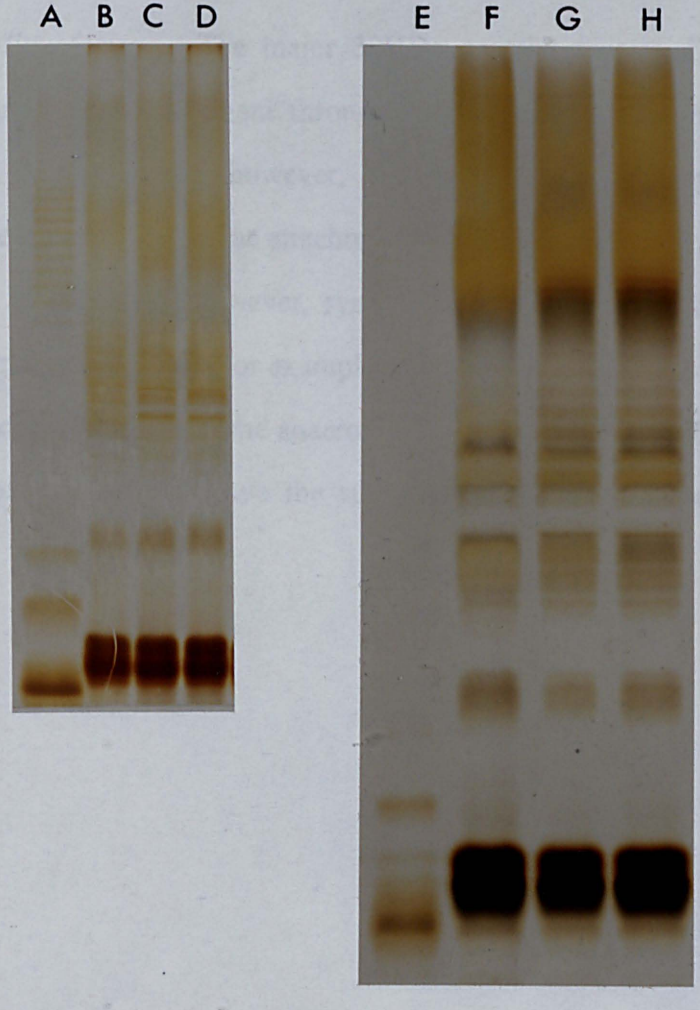
C: *S.liquefaciens* treated with active trypsin

D: *S.liquefaciens* treated with heat inactivated trypsin

F: *P.fragi* treated with active trypsin

G: *P.fragi* treated with heat inactivated trypsin

H: *P.fragi* untreated control



5.8 Effect of anaerobiosis

5.8.1 OMP profiles

The effect of anaerobic growth on *S.liquefaciens* OMPs is shown in Figure 5.13. The most obvious effect is the expression of the 32KDa protein under anaerobic growth conditions. A minor (46KDa) band was present only under aerobic conditions. Anoxic growth conditions have been reported to affect the OMP profiles in a variety of bacteria, at least three novel proteins are induced in *Neisseria gonorrhoea* when grown anaerobically (Clark *et al.*, 1987), growth of *Salmonella* sp under oxygen limited conditions repressed the expression of an outer membrane protein (Schieman and Shope, 1991) however Lee and Falkow (1990) showed that there was an increase of certain proteins in the same organism. Davies *et al.* (1992) studied the effect of aeration on OMP expression in *Pasturella haemolytica* and found that the OMPs varied with the degree of aeration.

The attachment results (chapter four) showed that attachment was higher in aerobically grown *S.liquefaciens*. The major 32KDa protein expressed under anaerobic growth conditions may affect attachment through indirect effects on hydrophobicity and charge (discussed in chapter seven), however, the results described in previous sections suggest that OMPs are not involved in the attachment process.

In the natural environment, however, synthesis of a protein that affects attachment may present an ecological advantage. For example, if a cell was attached to a surface and the micro environment around the cell became anaerobic, then reduction in the forces involved in attachment may enable the cell to leave the surface and therefore disperse into aerobic conditions.

Figure 5.13

SDS-PAGE showing the effect of anaerobiosis on the OMP profile of *S.liquefaciens*

S.liquefaciens was grown aerobically or anaerobically in LB broth to early stationary phase as described in the materials and methods.

Key to tracks

A: Protein markers

B: Aerobic (7µg protein)

C: Anaerobic (7µg protein)

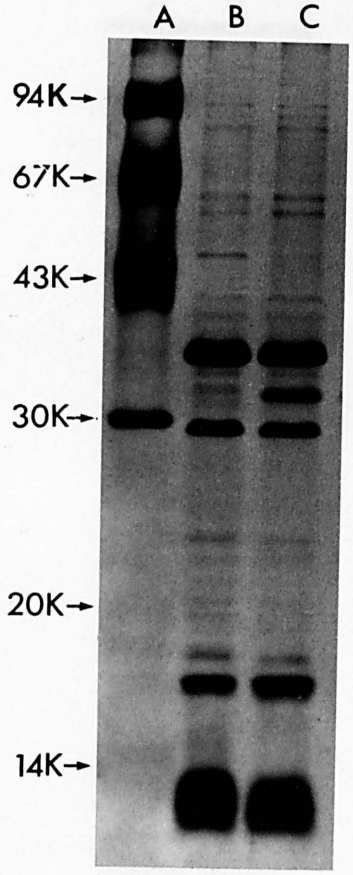


Figure 5.14

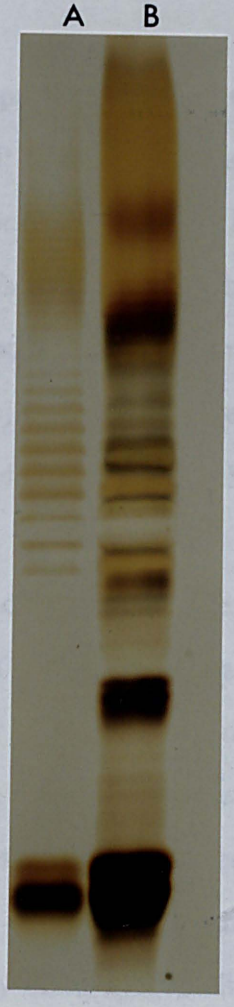
Modified SDS-PAGE showing the effect of anaerobiosis on the LPS profile of *S.liquefaciens*

S.liquefaciens was grown aerobically or anaerobically in LB broth and the outer membrane preparations adjusted to the same protein concentration were treated with proteinase K to extract the LPS and 6µl added to each track.

Key to tracks

A: Anaerobic

B: Aerobic



5.8.2 LPS profiles

Figure 5.14 shows that anaerobic growth also results in significant changes in the LPS profile most noticeably in the low molecular weight region. Davies *et al.* (1992) also found differences in the low mass components of LPS from *Pasteurella haemolytica* grown aerobically and anaerobically. Similarly Tsai *et al.* (1983) had found that the LPS composition of *Neisseria gonorrhoea* varied with aeration.

As already described, the attachment of *S.liquefaciens* was higher in aerobic cultures. It is possible therefore that the loss of various LPS components in addition to the protein changes observed in aerobic cultures reduce attachment through effects on hydrophobicity and charge (discussed in chapter seven).

5.9 Comparison of OMP profiles of attached and liquid phase cells

The differences in OMP profiles of attached and liquid phase *S.liquefaciens* are shown in Figure 5.15. *S.liquefaciens* was grown in LB broth to the mid exponential phase, and added to stainless steel surfaces for 2 hr. The surfaces were rinsed to remove loosely attached cells, and the attached population was removed from the surface by mild sonication. Liquid phase cells were also subjected to the same sonication so that both types of cells had the same treatment, this assumed that sonication had the same effect on attached cells as it had on those in liquid culture.

Figure 5.15 shows that certain differences were apparent. The liquid phase cells showed increased expression of 28 and 30 KDa proteins, whilst the attached population appeared to possess additional or increased levels of several high molecular weight proteins.

Although a pure culture of *S.liquefaciens* was used, this was not a homogeneous population as a range of cell types were present from newly formed daughter cells to elongated cells on the point of division. Allison *et al.* (1990b) found that newly formed daughter cells were significantly more hydrophilic than the biofilm cells from which they originated. These workers concluded that the dispersal of cells from surfaces and the recolonization of new surfaces reflected cell cycle mediated events.

Figure 5.15

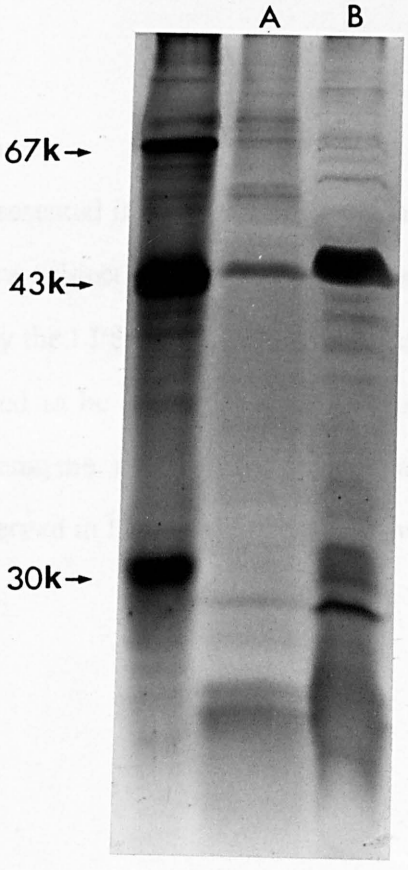
SDS-PAGE showing the OMP profiles of attached and liquid phase *S.liquefaciens*

Exponential phase *S.liquefaciens* was added to sterile stainless steel surfaces for 2 hr. The surfaces were rinsed to remove loosely attached cells and the attached population removed by mild sonication. The liquid phase culture was also subjected to the same sonication and the OMP isolated from the liquid phase and attached populations.

Key to tracks

A: *S.liquefaciens* - removed from the stainless steel surface by sonication

B: *S.liquefaciens* - liquid phase culture exposed to the same sonication



Chapter seven examines the possibility of a heterogeneous population with differing surface characteristics in more detail. It is, however, possible that the differences in OMP profiles are due to growth rate effects. The attached and liquid phase populations may have different nutrient availablities and therefore growth rates. Growth rates have been shown to influence OMP synthesis (Anwar *et al.*, 1991) and therefore the differences in OMP of attached and liquid phase populations may be related to the differing growth rates rather than attachment *per se*.

However, the OMP profiles would seem to suggest that heterogeneous populations (whether due to cell age and cell cycle effects or differing growth rates) may exist in pure culture systems and that differences between these sub-populations may result in differing attachment abilities and a partitioning of these sub-populations between the solid and liquid phases.

5.10 Conclusions

The results presented in this chapter show that the OMP and LPS profiles of *P.fragi* and *S.liquefaciens* are subject to considerable variation depending upon the environmental conditions. Generally the LPS profile of *S.liquefaciens* showed greater variability.

There appeared to be no correlation between attachment and OMP profiles, which was confirmed by enzyme modification of the outer cell envelope. Although certain differences were observed in the LPS profiles there was no obvious correlation to attachment.

CHAPTER SIX

6. EXOPOLYSACCHARIDES (EPS)

6.1 Introduction

Many bacterial species have been shown to produce or release polymers but the ecological significance of these polymers is not completely understood. Several reports have indicated that the polymers enhance adhesion of the bacterial cell to surfaces (Geesey, 1982; Rutter, 1980; Costerton *et al.*, 1981) and the involvement of exopolysaccharides (EPS) in bacterial attachment has been documented for freshwater (Sutherland, 1980) and marine bacteria (Floodgate, 1972), and more recent work has examined the involvement of these polymers in the attachment of bacteria to food contact surfaces (Zottola, 1991). There are few reports which distinguish between polymers involved in initial adhesion and those involved in the subsequent colonization process. Allison and Sutherland (1987) demonstrated that a non-polysaccharide producing mutant attached to surfaces in similar numbers to the wild type but was unable to form microcolonies on the surface. These workers concluded that the EPS was involved in film development but was not required for the initial attachment process. The various polymers are also thought to act as a protective barrier against phagocytes, bacteriophages, antibiotics, biocides, surfactants and antibodies (Costerton *et al.*, 1985).

The apparent involvement of EPS in the non-specific attachment to solid surfaces was first suggested by Zobell (1943) and has since been supported by numerous electron microscopy studies (Marshall *et al.*, 1971; Fletcher and Floodgate, 1973; Costerton *et al.*, 1981, 1985; Zottola, 1991). However, Fletcher (1980) treated cells with periodate, which reacts with EPS and demonstrated that attachment of these cells was inhibited.

Previous studies have shown that many parameters can influence the synthesis of EPS by bacteria (Duguid and Wilkinson, 1953; Read and Costerton, 1987). Carbohydrate and nitrogen nutrition, cofactors, temperature and aeration can influence microbial EPS production in different ways in different species e.g. higher than optimal growth temperatures increased EPS production in *Xanthomonas campestris* B1459 (Shu and Yang, 1990) but in *Klebsiella aerogenes* lower than optimal temperature resulted in maximal EPS production

(Duguid and Wilkinson, 1953). The influence of nitrogen limitation also varies between strains increasing EPS production by *Xanthomonas campestris* B1459 (Souw and Demain, 1979) and *Zoogloea ramigera* (Norberg and Enfors, 1982), but decreasing EPS synthesis in *Porphyridium* sp strain UTEX637 (Arad *et al.,* 1988), *Haloferax mediterranei* (Anton *et al.*, 1988) and *Butyrivibrio fibrisolvens* nyx (Wachenheim and Patterson, 1992). Therefore the effect of the growth conditions on EPS production must be determined individually for the species involved.

6.2 Aims

Growth conditions influence EPS production and the aim of this section was to determine the effect of nutrient limitation, temperature, pH, anaerobiosis and growth phase on the production of EPS by *S.liquefaciens, S.cohnii* and *P.fragi.* The attachment data described in chapter 4 is discussed in the light of these findings. The effect of surface growth on EPS production was also examined.

6.3 Comparison of extraction methods

Various methods have been used to isolate bacterial exopolysaccharide ranging from simple centrifugation and dialysis extractions to complex capsule preparations. Stack (1988) and Ha *et al.* (1991) describe isolations based on the dialysis of cell free supernatants. Various workers have used solvents to precipitate the polysaccharide, followed by dialysis. The solvents used include ice cold ethanol (Anton *et al.*, 1988; Racine *et al.*, 1991; Wachenheim and Patterson, 1992), acetone (Kennedy and Sutherland, 1987; Allison and Sutherland, 1987) and isopropanol (Beech *et al.*, 1991) and the amount of exopolysaccharide isolated by these methods has been quantified by weight, total sugar or uronic acid assays. Uronic acids are unique to the polymers found outside the cytoplasmic membrane of cells and are therefore useful specific indicators of extracellular polymers (Fazio *et al.*, 1982).

Light and transmission / scanning electron microscopy studies have shown that the exopolysaccharides produced by *S.liquefaciens, P.fragi* and *S.cohnii* are loosely bound to the cell surface rather than of the discrete capsular type exopolysaccharide (Figures 6.1-6.3).

Figure 6.4 (a and b) compares the total sugar and uronic acid content of polysaccharides isolated by two different methods; dialysis (Ha *et al.*, 1991) and isopropanol precipitation (Beech *et al.*, 1991). This data shows that the isopropanol precipitation method consistently resulted in higher yields of exopolysaccharide as measured by total sugar and uronic acid assays. The fact that the uronic acid data mirrors the total sugar results suggests that little cellular contamination is present.

The data also shows that *S.cohnii* and *P.fragi* produce 4-6 times higher levels of EPS than *S.liquefaciens.* The addition of 0.1% (v/v) dialysate of yeast extract slightly increased the EPS yields.

6.4 Effect of growth pH

Figure 6.5 shows the EPS produced by *S.liquefaciens, P.fragi* and *S.cohnii* at different pHs in nitrogen limited media. For all three species, EPS production (as measured by total sugar and uronic acid) was lowest at pH 7.5 and higher at 6.5 and 8.5. The growth pH optima are 7.5-8 for *S.liquefaciens*, 6.5-7 for *P.fragi* and 7.5-8 for *S.cohnii* (Figure 3.2). Consequently maximal EPS production does not correlate to optimal biomass production. Racine *et al.* (1991) also found that EPS production by *Propionibacterium acidi-propionici* VM-25 was not maximal at the optimum growth pH, although the pH at which EPS production was maximal was in this case pH 6.0.

It has been suggested that EPS synthesis may increase under stress conditions. Wrangstadh *et al.* (1986) found that *Pseudomonas* sp S9 produced and released an extracellular polysaccharide during complete energy and nutrient starvation. Consequently increased EPS production at pH other than the optimum may be due to a similar stress response.

Figure 6.1

Nitrogen limited *S.liquefaciens* grown on glass coverslips and stained with congo red (Magnification 1000x)

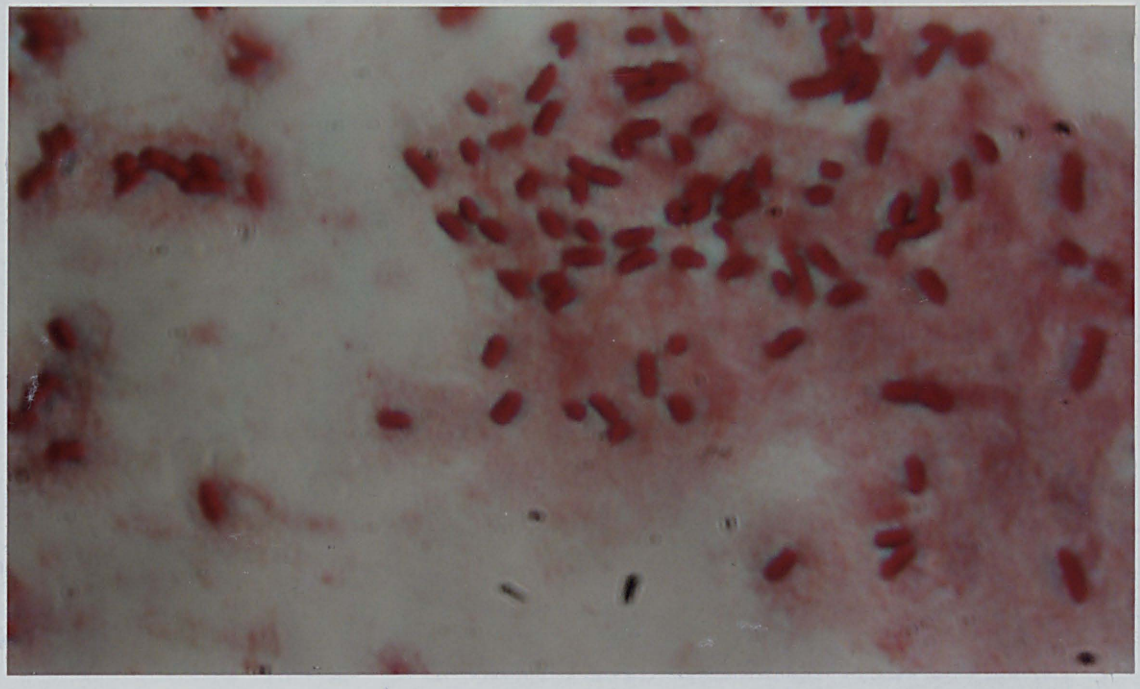
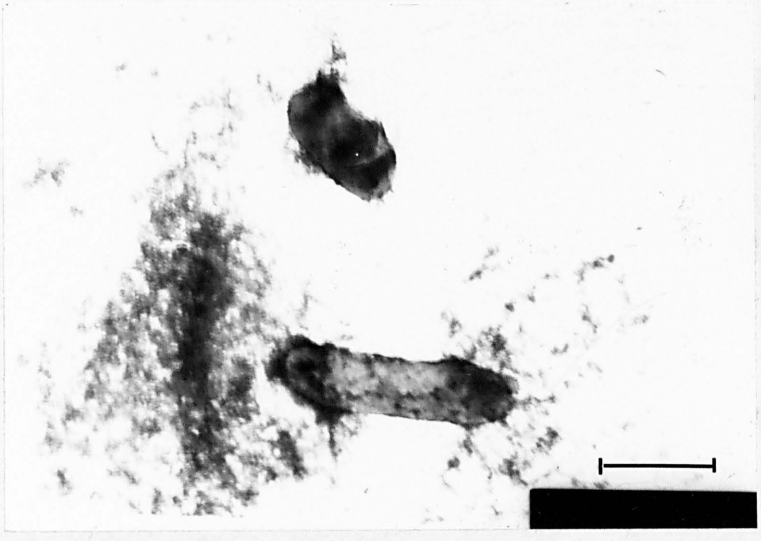


Figure 6.2

Transmission electron micrograph of negatively stained *P.fragi*



P.fragi was negatively stained with phosphotungstic acid as described in section 2.17.1. Scale bar = 1μm.

Figure 6.3

Scanning electron micrograph of a mixed population biofilm

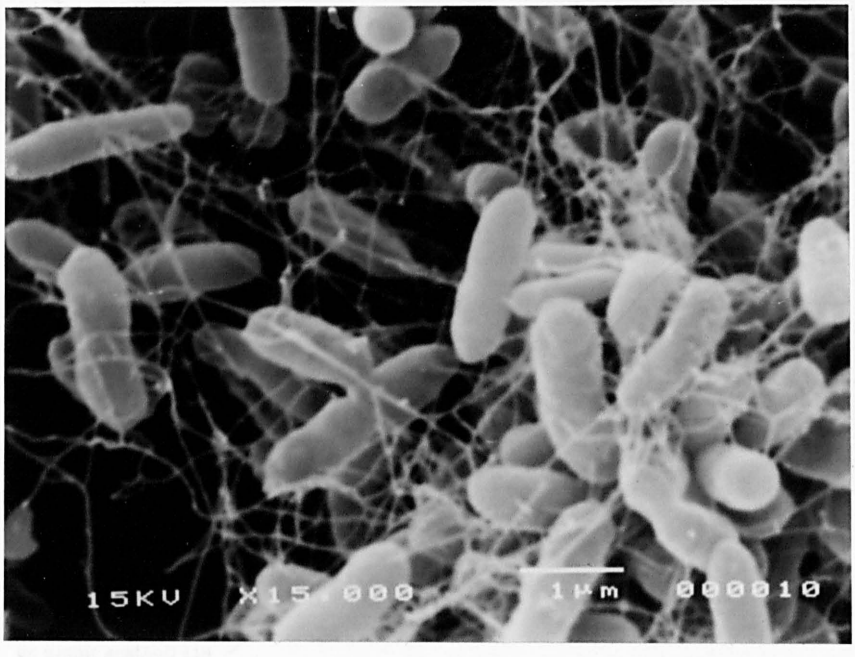
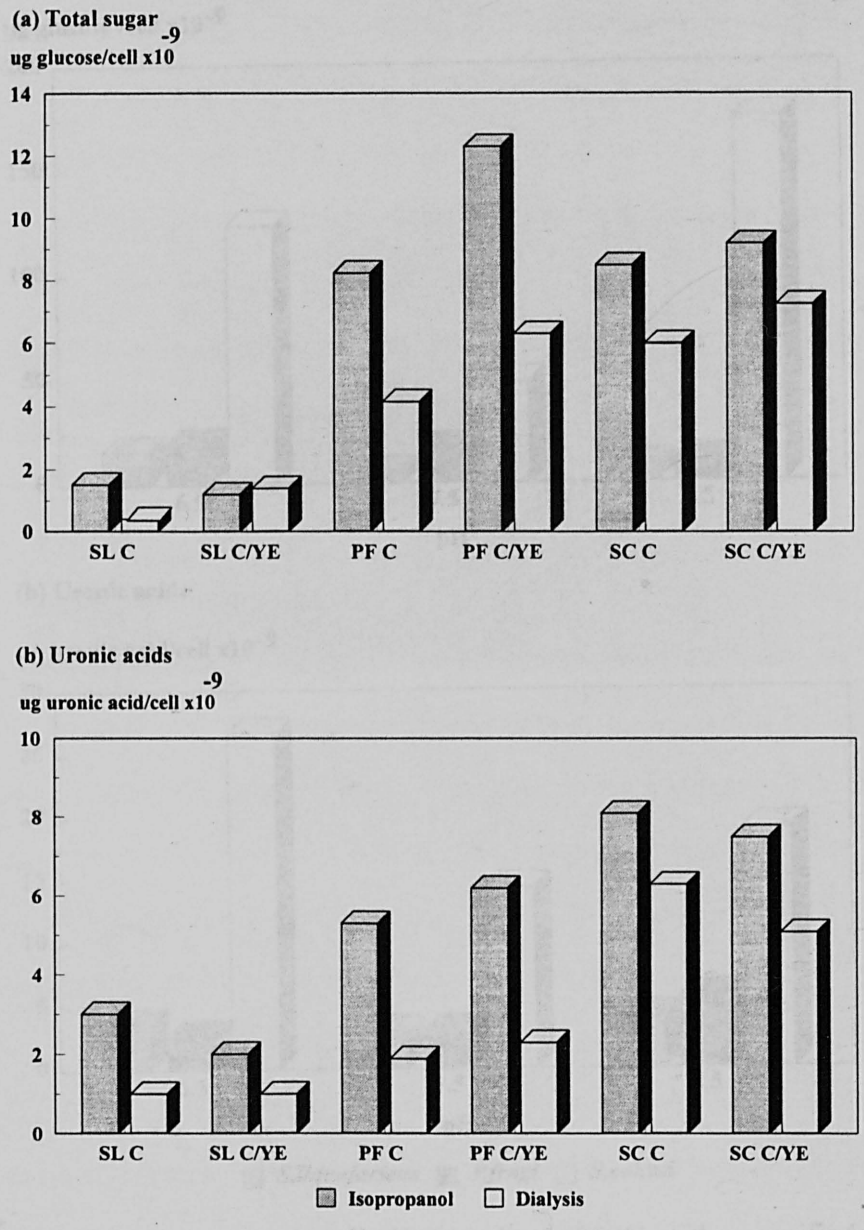


Figure 6.4

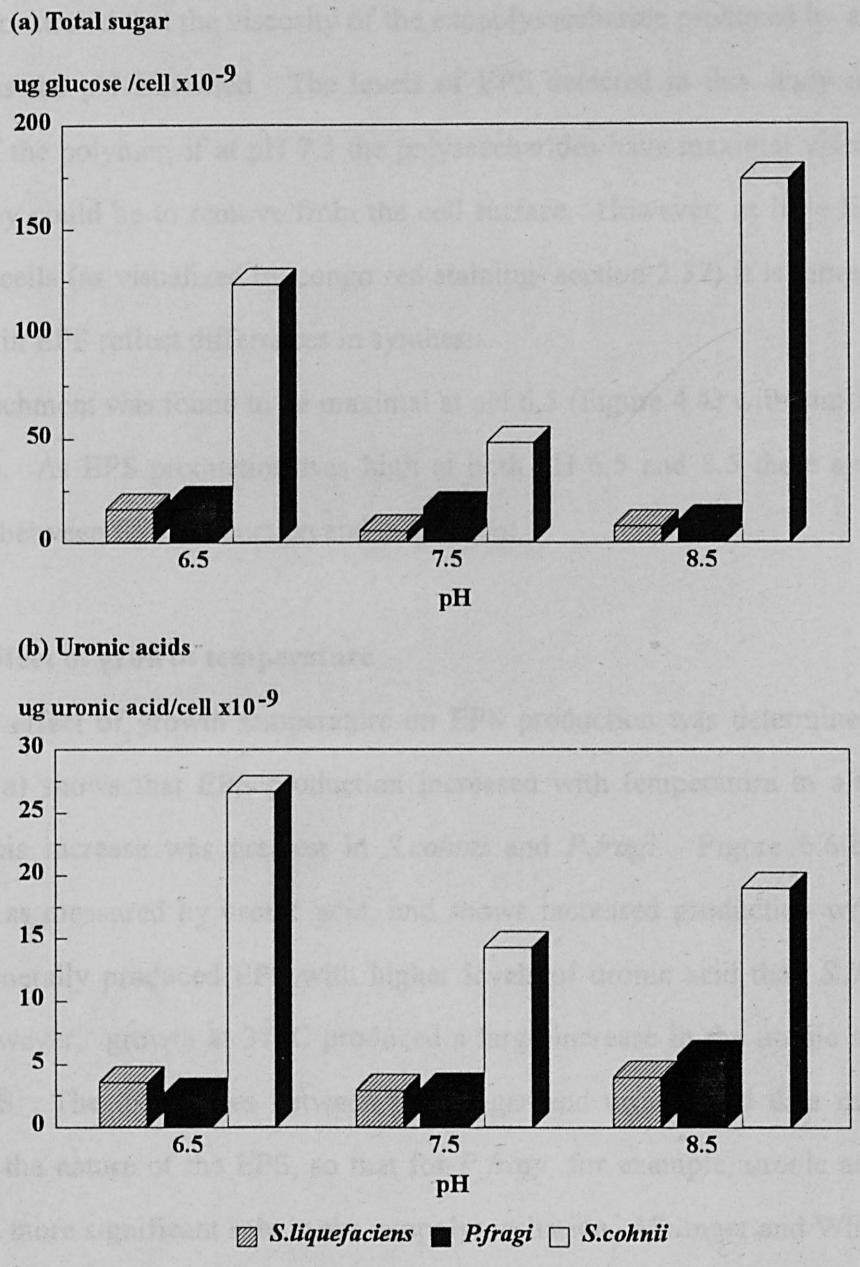
Comparison of EPS extraction methods



The bacteria were grown in carbon excess medium for 16 hr at 25°C, and the EPS produced was isolated by isopropanol (section 2.33.2) or dialysis (section 2.33.1). The EPS was quantified by (a) total sugar and (b) uronic acid assays.

Figure 6.5

Effect of growth pH on EPS production



The bacteria were grown in nitrogen limited media at 25°C at the specified pHs. EPS was isolated using isopropanol precipitation (section 2.33.2) and quantified by (a) total sugar and (b) uronic acid assays.

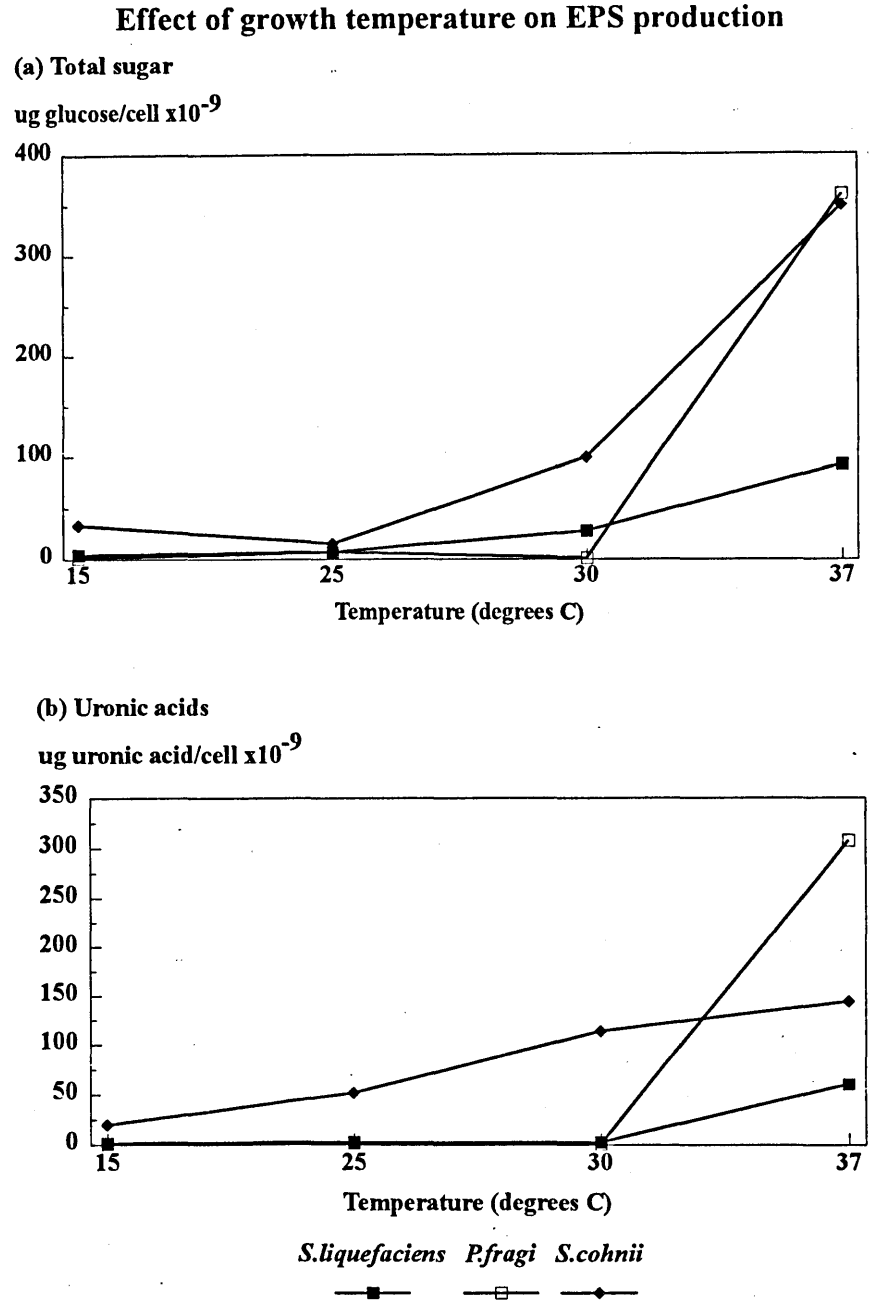
Sutherland (1983) has shown that the exopolysaccharide produced by a freshwater bacterium had maximum viscosity at pH 7, and decreased at pH 3 and 11. Boyle and Read (1983) demonstrated that the viscosity of the exopolysaccharide produced by a marine isolate decreased as the pH increased. The levels of EPS detected in this study may reflect the viscosity of the polymer, if at pH 7.5 the polysaccharides have maximal viscosity, the more difficult they could be to remove from the cell surface. However, as little EPS was visible around the cells (as visualized by congo red staining- section 2.37) it is more likely that the differences in EPS reflect differences in synthesis.

Attachment was found to be maximal at pH 6.5 (Figure 4.4) with similar levels at pH 7.5 and 8.5. As EPS production was high at both pH 6.5 and 8.5 there appears to be no correlation between EPS production and attachment.

6.5 Effect of growth temperature

The effect of growth temperature on EPS production was determined (Figure 6.6). Figure 6.6(a) shows that EPS production increased with temperature in all three species, although this increase was greatest in *S.cohnii* and *P.fragi*. Figure 6.6(b) depicts EPS production as measured by uronic acid, and shows increased production with temperature. *S.cohnii* generally produced EPS with higher levels of uronic acid than *S.liquefaciens* and *P.fragi*, however, growth at 37°C produced a large increase in the uronic acid detected in *P.fragi* EPS. The differences between total sugar and uronic acid data may well reflect changes in the nature of the EPS, so that for *P.fragi*, for example, uronic acid components may play a more significant role in the exopolysaccharide. Uhlinger and White (1983) have reported that maximal formation of uronic acid rich EPS occured during periods of metabolic stress.

Figure 6.6



The bacteria were grown in nitrogen limited medium at the specified temperatures for 16 hr. EPS was isolated by isopropanol extraction (section 2.33.2) and quantified by (a) total sugar and (b) uronic acid assays.

The possibility that temperature may influence the nature and composition of the EPS was examined by thin layer chromatography of the neutral sugars present in partially purified EPS (section 2.33.3). Figure 6.7 shows the sugars present in EPS isolated from cells grown at different temperatures, and Table 6.1 summarises the sugars detected at each temperature. Although it was extremely difficult to obtain sufficient hydrolysed EPS to spot onto the plate, certain differences can be seen e.g. fucose was detected in *S.liquefaciens* EPS at 15 and 25 °C but was not at 30 and 37°C; rhamnose was not detected in *P.fragi* EPS from cells grown at 25°C.

The three species have different optimum growth temperatures; *S.liquefaciens*, 30°C, *S.cohnii*, 25°C and *P.fragi*, 37°C. Only *P.fragi*, therefore, produced maximal amounts of EPS at the optimum growth temperature.

Growth temperature has been reported to have variable effects on EPS production. Several workers have found that EPS production was maximal at temperatures below the optimum growth temperature (Duguid and Wilkinson, 1953; Williams and Wimpenny, 1977; Manresa *et al.*, 1987; Graber *et al.*, 1988; Racine *et al.*, 1991), however, Shu and Yang (1990) found that higher than optimal growth temperatures resulted in increased EPS production in *Xanthomonas campestris*.

The attachment of *S.liquefaciens, P.fragi* and *S.cohnii* at different temperature was examined in section 4.3.1.4. Maximal attachment was observed with cells grown at 37°C. As maximal EPS was also observed at 37°C in all three organisms, it would appear that there is a correlation between EPS production and attachment. Other workers have found varied relationships between exopolysaccharides production and attachment, for example EPS production has been shown to enhance the attachment of marine bacteria to surfaces (Costerton *et al.*, 1981; Fletcher and Floodgate, 1973, Zottola, 1991), however, EPS has also been shown to inhibit (Pringle and Fletcher, 1983; Rosenberg *et al.*, 1983; Wrangstadh *et al.*, 1986) or have no effect on the attachment process (Allison and Sutherland, 1987).

Figure 6.7

TLC showing the effect of growth temperature on the neutral sugars present in EPS

EPS isolated from cells grown at the specified temperatures was partially purified (section 2.33.3) and separated by TLC (2.36).

	Standards		Samples
1	Fucose	A	*S.cohnii* 15 °C
2	Galactose	B	*S.cohnii* 25 °C
3	Glucose	C	*S.cohnii* 30 °C
4	Mannose	D	*S.cohnii* 37 °C
5	Ribose	E	*P.fragi* 15 °C
6	Rhamnose	F	*P.fragi* 25 °C
7	Xylose	G	*P.fragi* 30 °C
		H	*P.fragi* 37 °C
		I	*S.liquefaciens* 15 °C
		J	*S.liquefaciens* 25 °C
		K	*S.liquefaciens* 30 °C
		L	*S.liquefaciens* 37 °C

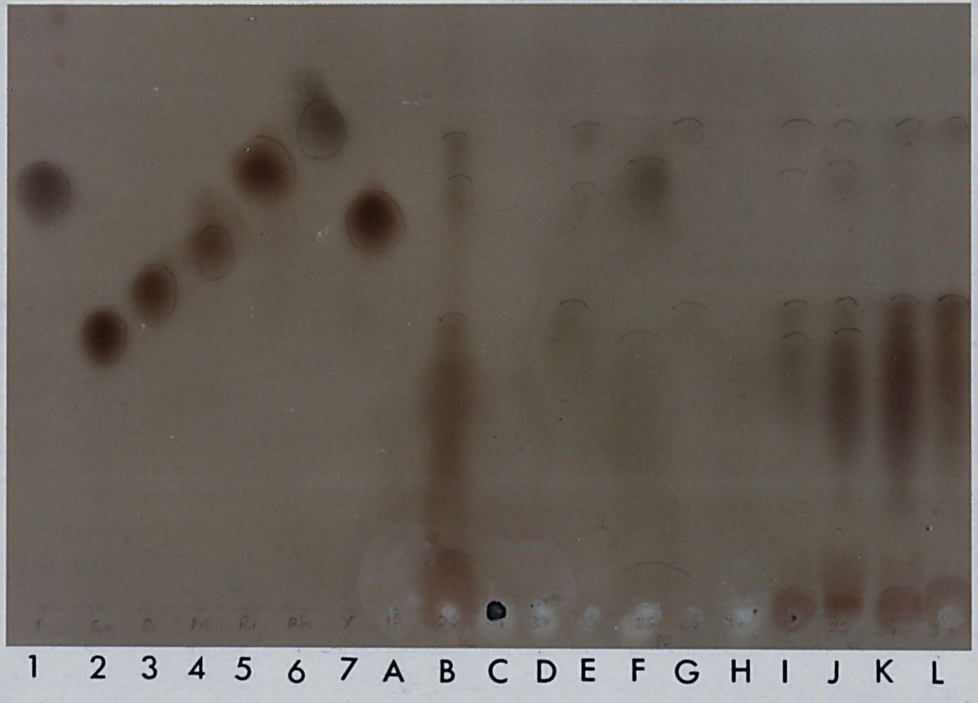


Table 6.1

Summary of the neutral sugars detected in the EPS isolated from cells grown at different temperatures

Organism	Temp (^{0}C)	Neutral sugars detected
S.cohnii	15	*
	25	Rhamnose, Xylose, Galactose
	30	*
	37	*
P.fragi	15	Galactose, Xylose, Rhamnose
	25	Galactose, Fucose
	30	Galactose, Rhamnose
	37	*
S.liquefaciens	15	Galactose, Glucose, Fucose, Rhamnose
	25	Galactose, Glucose, Fucose, Rhamnose
	30	Galactose, Glucose, Rhamnose
	37	Galactose, Glucose, Rhamnose

* Below detection limit

6.6 Effect of nutrient limitation

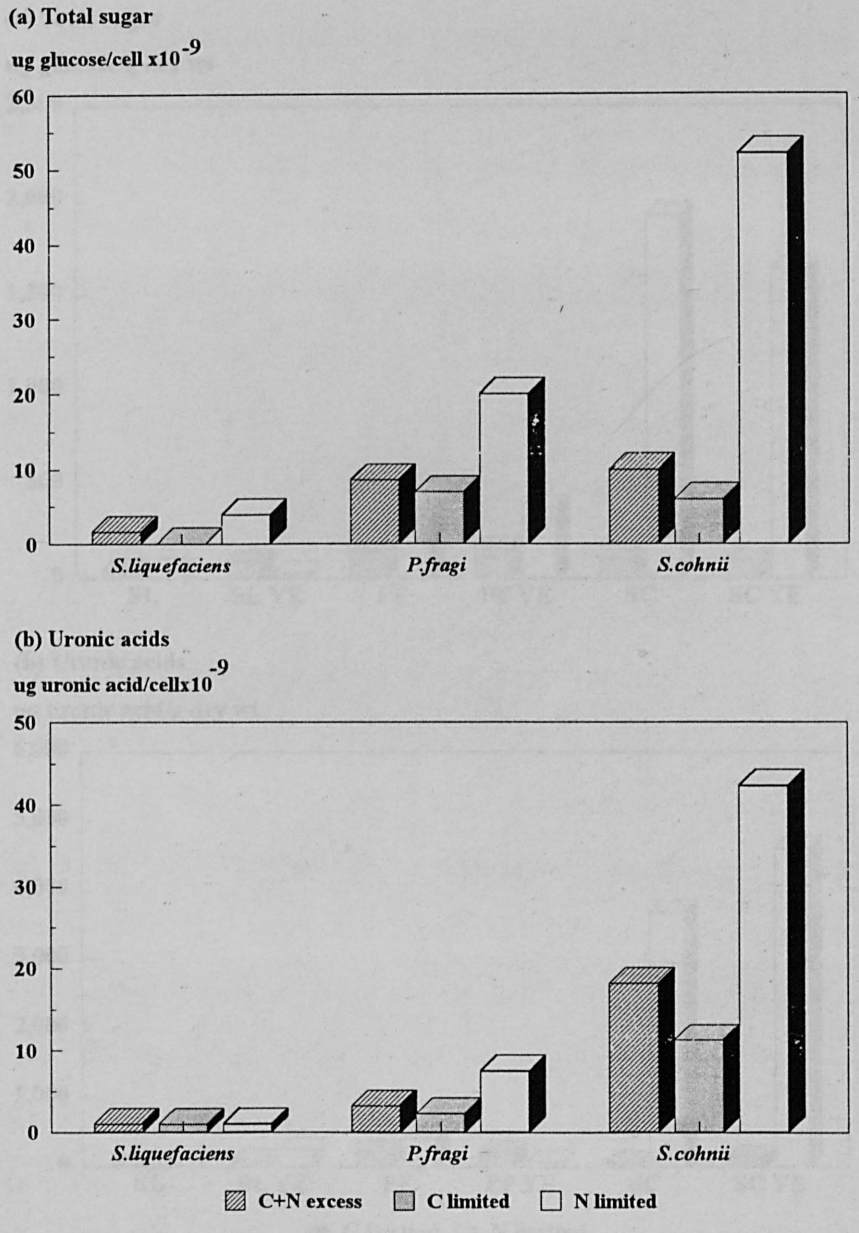
Figure 6.8 (a and b) shows the effect of nutrient limitation on EPS production by *S.liquefaciens*, *P.fragi* and *S.cohnii*. Carbon and nitrogen limitation were chosen as appropriate variables as nitrogen limitation appears to be a common factor controlling EPS synthesis, and because carbon and nitrogen limitations are likely to be encountered in the natural environment. Figure 6.8(a) shows that with carbon and nitrogen excess, *S.cohnii* and *P.fragi* produce similar amount of EPS and higher levels than *S.liquefaciens*. The same relationship was found as under carbon limitation although the amounts produced by each organism were lower. Nitrogen limitation resulted in a marked increase in EPS production; two fold in *P.fragi*, and five fold in *S.cohnii*.

As well as calculating micrograms of glucose or uronic acid per cell, the amount of EPS present was calculated per gram dry weight (Figure 6.9). This shows the differences in EPS production by carbon and nitrogen limited cells. The most obvious effect is the significantly higher levels of EPS produced by the nitrogen limited cells, particularly *P.fragi* and *S.cohnii*. The increase observed for *S.cohnii* appears to be more marked when the data is presented as sugar per g dry weight, however calculations per cell were per viable cell and therefore did not include non viable cells.

The production of EPS under carbon and nitrogen limitation was visualized using congo red staining (Allison and Sutherland, 1984 - section 2.37). Figures 6.10 to 6.12 show *S.cohnii*, *S.liquefaciens* and *P.fragi* attached to glass coverslips and grown under carbon (a) or nitrogen (b) limitation. In all cases, polysaccharide is obviously visible around the cells grown under nitrogen limitation and difficult to detect in carbon limited cells; thus confirming the chemical assays. Figure 6.13 shows a mixed culture of the three species attached to a glass coverslip and grown under carbon (a) or nitrogen (b) limitation. Polysaccharide material is visible around the mixed species microcolonies grown under nitrogen limitation and appears to be denser than that observed around single species.

Figure 6.8

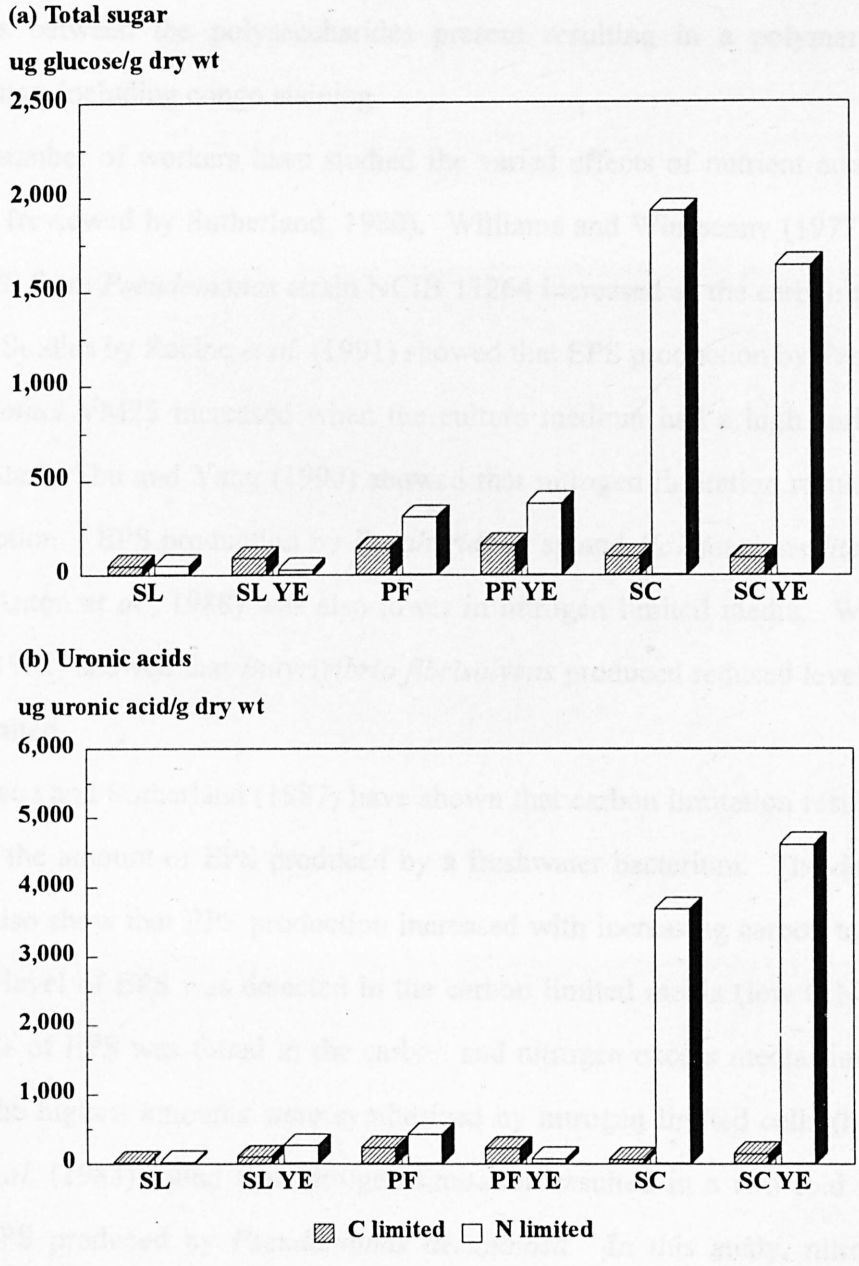
Effect of nutrient limitation on EPS production calculated per viable cell



The bacteria were grown in the specified media for 16 hr at 25°C. EPS was extracted by isopropanol precipitation (section 2.33.2) and quantified by calculating (a) total sugar and (b) uronic acid present per cell.

Figure 6.9

Effect of nutrient limitation on EPS production calculated per gram dry weight



The bacteria were grown in the specified media for 16 hr at 25°C. EPS was extracted by isopropanol precipitation (section 2.33.2) and quantified by calculating (a) total sugar and (b) uronic acid present per gram dry weight.

Allison (1992) demonstrated that a mixture of polymers may have substantially higher viscosity than the individual polymers, therefore it is possible that the difference in the mixed species polysaccharide matrix is not an artefact of the staining but due to the interactions between the polysaccharides present resulting in a polymer with different characteristics, including congo staining.

A number of workers have studied the varied effects of nutrient conditions on EPS production (reviewed by Sutherland, 1980). Williams and Wimpenny (1977) found that the yield of EPS from *Pseudomonas* strain NCIB 11264 increased as the carbon to nitrogen ratio increased. Studies by Racine *et al.* (1991) showed that EPS production by *Propionibacterium acidi-propionici* VM25 increased when the culture medium had a high carbon to nitrogen ratio. Similarly Shu and Yang (1990) showed that nitrogen limitation resulted in increased EPS production. EPS production by *Porphyridium* sp and *Haloferax mediterranei* (Arad *et al.*, 1988; Anton *et al.*, 1988) was also lower in nitrogen limited media. Wachenheim and Patterson (1992) showed that *Butyrivibrio fibrisolvens* produced reduced levels of EPS when nitrogen limited.

Allison and Sutherland (1987) have shown that carbon limitation results in a ten fold decrease in the amount of EPS produced by a freshwater bacterium. The data described in this study also show that EPS production increased with increasing carbon to nitrogen ratio. The lowest level of EPS was detected in the carbon limited media (low C:N ratio), slightly higher levels of EPS was found in the carbon and nitrogen excess media (intermediate C:N ratio) and the highest amounts were synthesized by nitrogen limited cells (high C:N ratio). Ombaka *et al.* (1983) found that nitrogen limitation resulted in a two fold increase in the levels of EPS produced by *Pseudomonas aeruginosa.* In this study, nitrogen limitation increased EPS production in *P.fragi* two fold, and EPS production by *S.cohnii* was five fold higher than the carbon limited level.

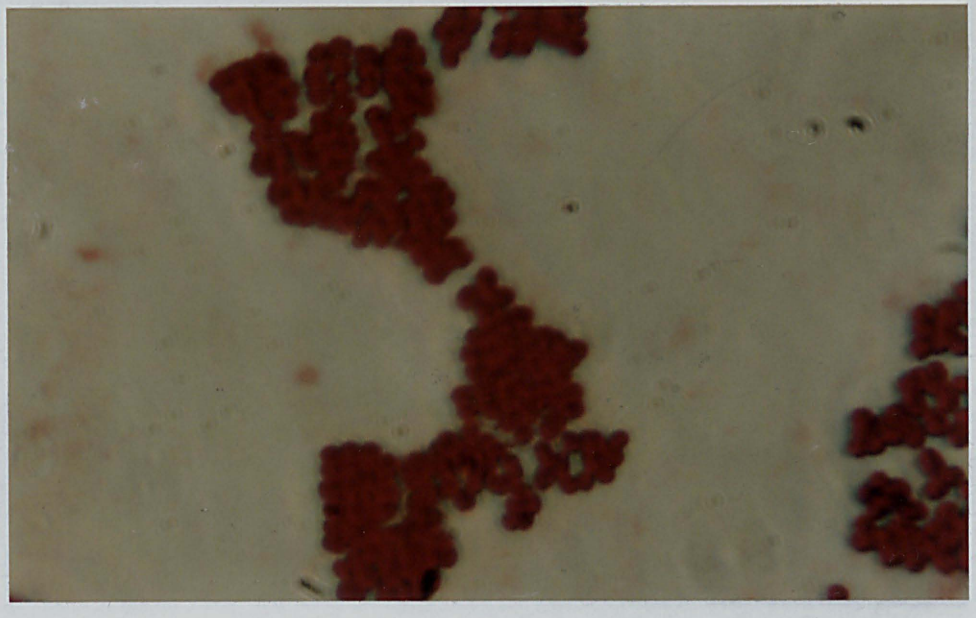
Phosphorous, sulphur and potassium deficiencies have also been reported to enhance EPS formation (Robinson *et al.*, 1984), whilst excess phosphate is thought to reduce EPS production (Bengtsson, 1991).

Figure 6.10

Congo red staining of *S.cohnii* attached to glass grown under (a) carbon and (b) nitrogen limitation.

S.cohnii was grown under carbon or nitrogen limitation, allowed to attach to glass coverslips for 6 hr and stained with congo red for polysaccharide (section 2.37). Magnification 1000x

(a)



(b)

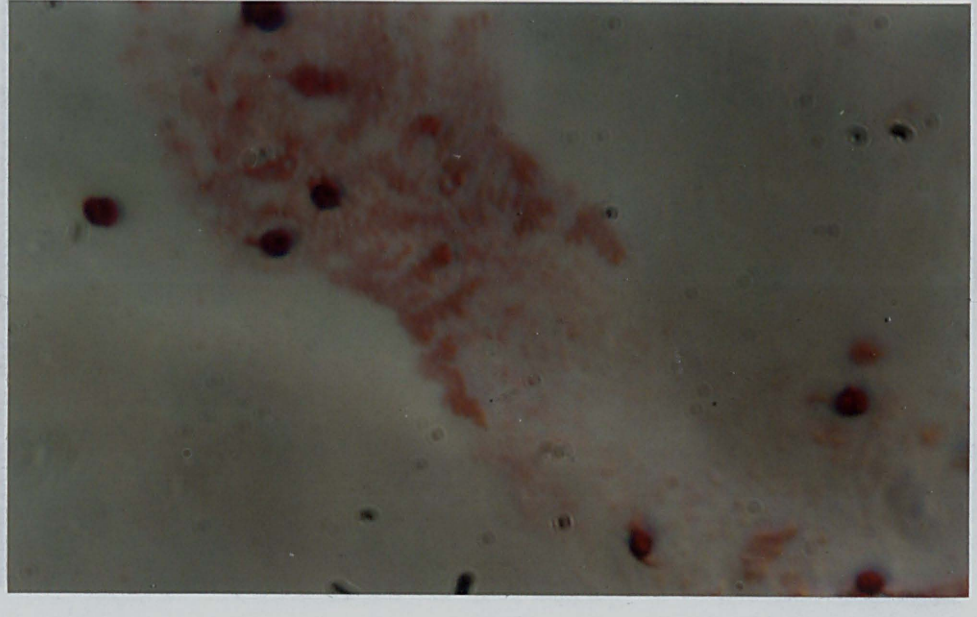
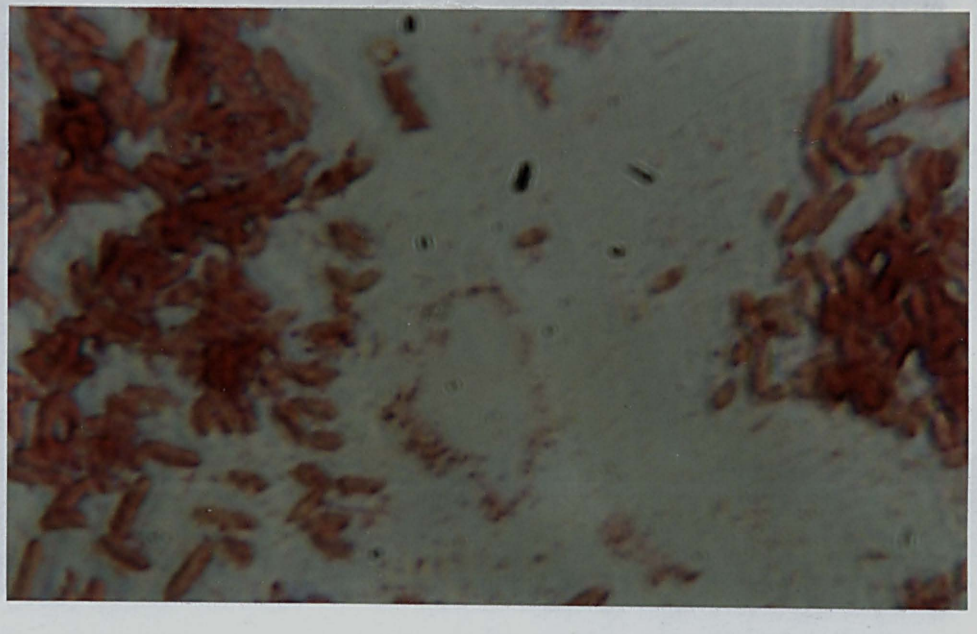


Figure 6.11

Congo red staining of *S.liquefaciens* attached to glass grown under (a) carbon and (b) nitrogen limitation.

S.liquefaciens was grown under carbon or nitrogen limitation, allowed to attach to glass coverslips for 6 hr and stained with congo red for polysaccharide (section 2.37). Magnification 1000x.

(a)



(b)

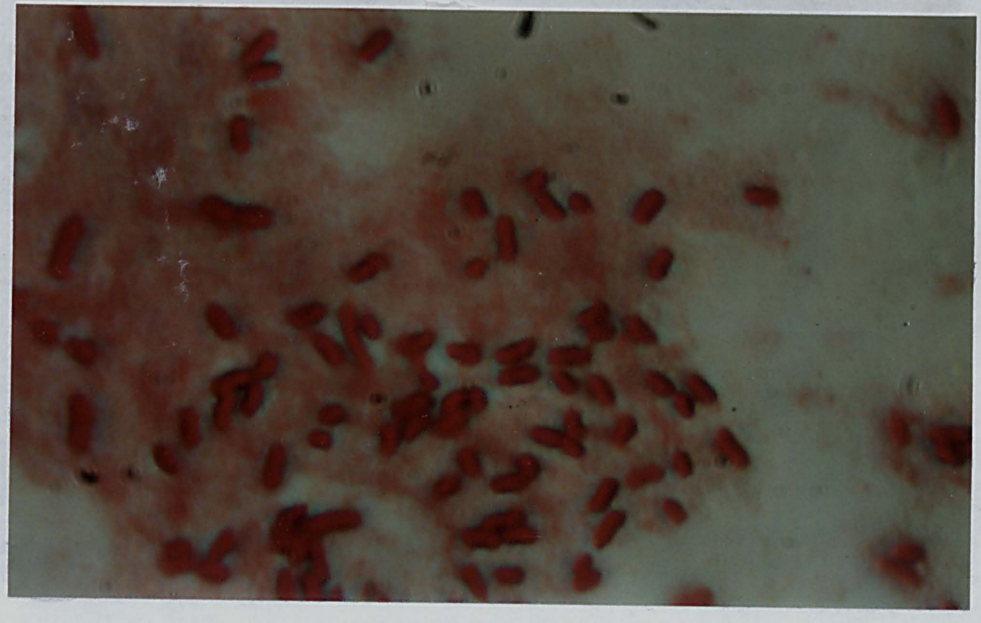
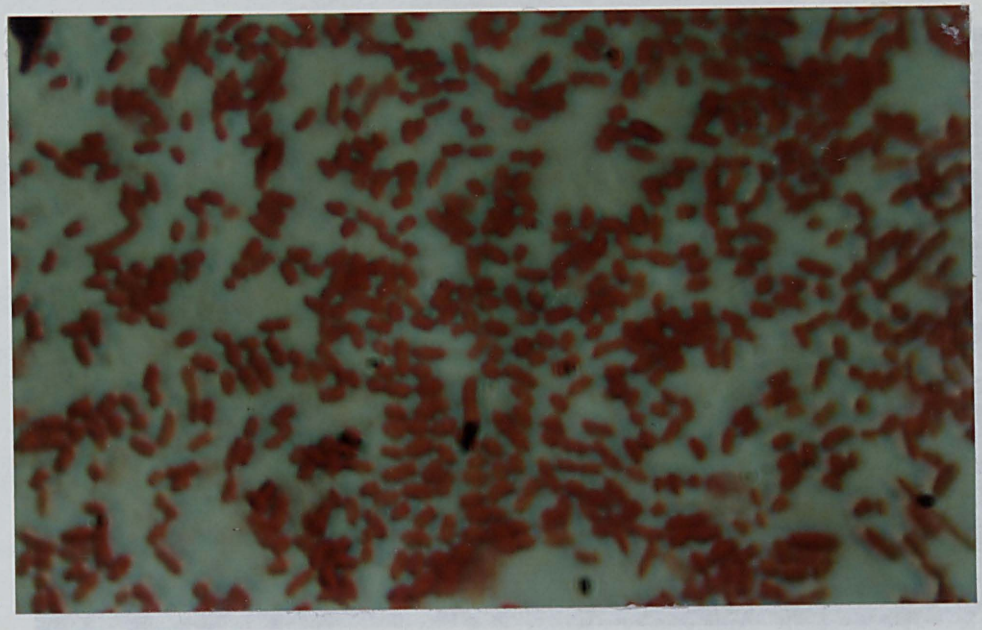


Figure 6.12

Congo red staining of *P.fragi* attached to glass grown under (a) carbon and (b) nitrogen limitation.

P.fragi was grown under carbon or nitrogen limitation, allowed to attach to glass coverslips for 6 hr and stained with congo red for polysaccharide (section 2.37). Magnification 1000x.

(a)



(b)

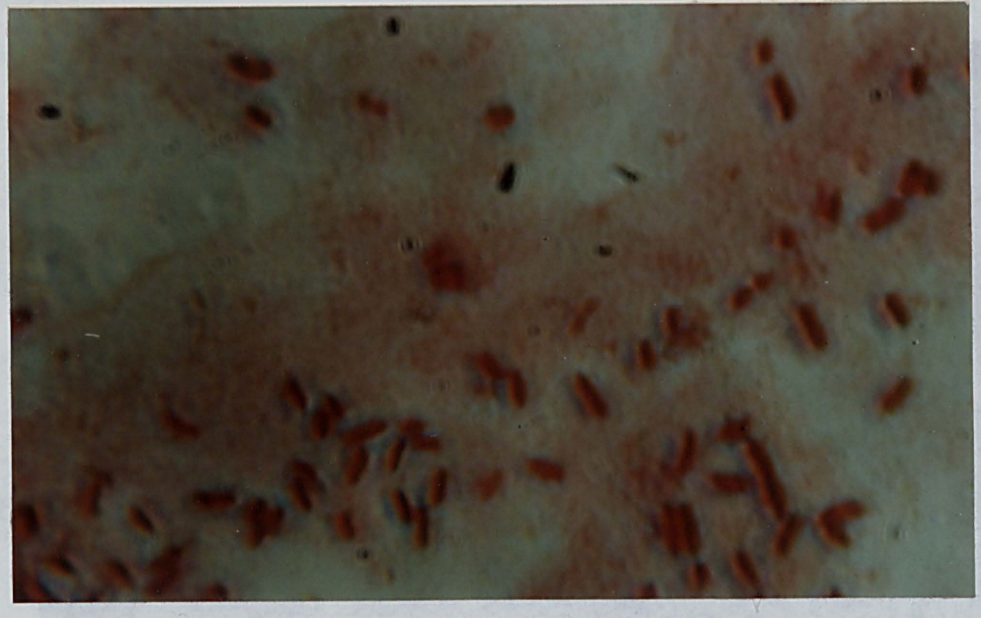
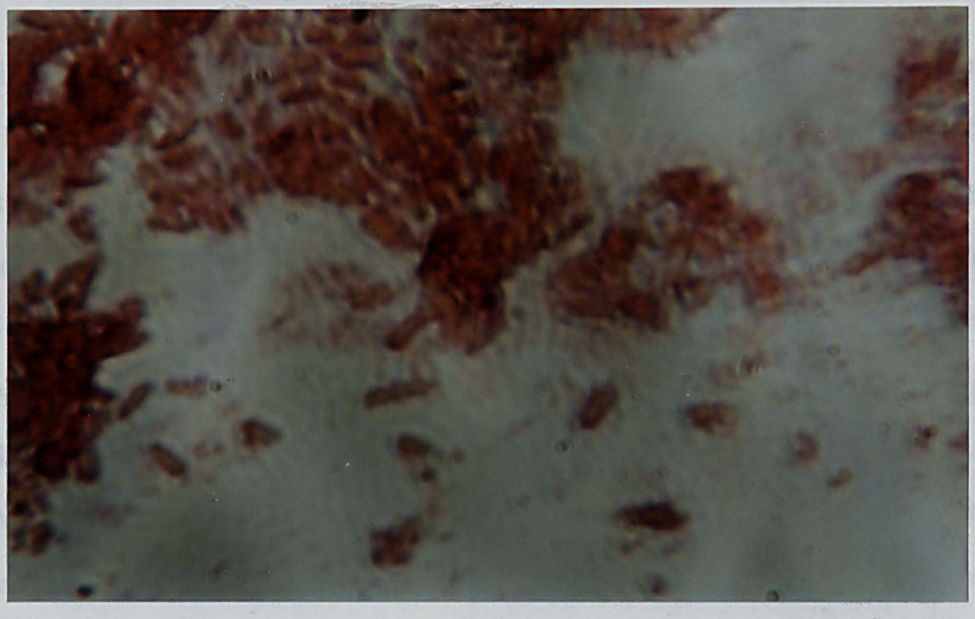


Figure 6.13

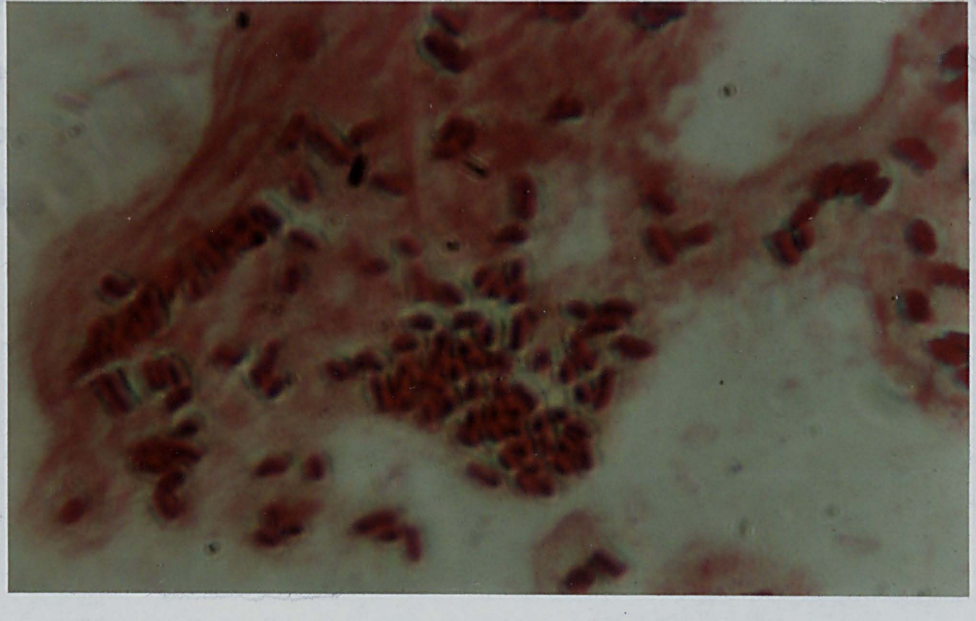
Congo red staining of a mixed culture biofilm attached to glass grown under (a) carbon and (b) nitrogen limitation.

The bacteria were grown in carbon or nitrogen limited mixed culture, allowed to attach to glass coverslips for 6 hr and stained with congo red for polysaccharide (section 2.37). Magnification 1000x.

(a)



(b)



The effect of the nature of the carbon source on EPS yield was not investigated in this study but various workers have reported that maximal yields are obtained using glucose as the carbon and energy source e.g. Rodrigues and Bhosle (1991) found higher yields when glucose was used as the sole carbon and energy source. Similarly Anton *et al.* (1988) showed that EPS production by *Haloferax mediterranei* was higher when glucose was the carbon and energy source. It is thought that additional metabolic steps may be required in EPS synthesis from different carbon sources which may influence production rates (Linton, 1990).

Figure 6.14 shows the neutral sugars present in EPS from cells under carbon and nitrogen limitation and Table 6.2 summarizes this data. Although, the spots are faint it is clear that there are differences in the nature of the EPS with nutrient limitation. The nutrient status and growth phase are thought to influence the quantity and chemical composition of the EPS produced (Decho, 1990). The nature of the polysaccharide produced by *Xanthomonas juglandis* under carbon limitation may differ from the polysaccharide produced under other growth conditions (Evans *et al.*, 1978), however, Bengtsson (1991) found that the exopolymers produced by ground water bacteria studied did not vary with growth condition and consisted of rhamnose, arabinose, mannose, galactose, galacturonic acid, glucose and glucuronic acid. In contrast, Uhlinger and White (1983) examined the relationship between physiological status and formation of EPS by *Pseudomonas atlantica* and found that the composition of the EPS varied through the growth cycle. Beech *et al.* (1991) found that the EPS isolated from free and attached cells differed in composition. All of these studies highlight the variability in terms of both quantity and composition of EPS.

Figure 6.14

TLC showing the variations in neutral sugars with nutrient limitation

The bacteria were grown in the specified media. EPS was isolated by isopropanol precipitation and partially purified (section 2.33.3) for TLC analysis (section 2.36). Dialysate of yeast extract (YE) was added at 0.1% (v/v) (section 2.4.2.4).

	Standards		Samples
1	Glucose	A	*P.fragi* carbon limited
2	Rhamnose	B	*P.fragi* carbon limited, with YE
3	Mannose	C	*P.fragi* nitrogen limited
4	Galactose	D	*P.fragi* nitrogen limited, with YE
5	Ribose	E	*S.cohnii* carbon limited
6	Fucose	F	*S.cohnii* carbon limited, with YE
7	Xylose	G	*S.cohnii* nitrogen limited
		H	*S.cohnii* nitrogen limited, with YE
		I	*S.liquefaciens* carbon limited
		J	*S.liquefaciens* carbon limited, with YE
		K	*S.liquefaciens* nitrogen limited, with YE

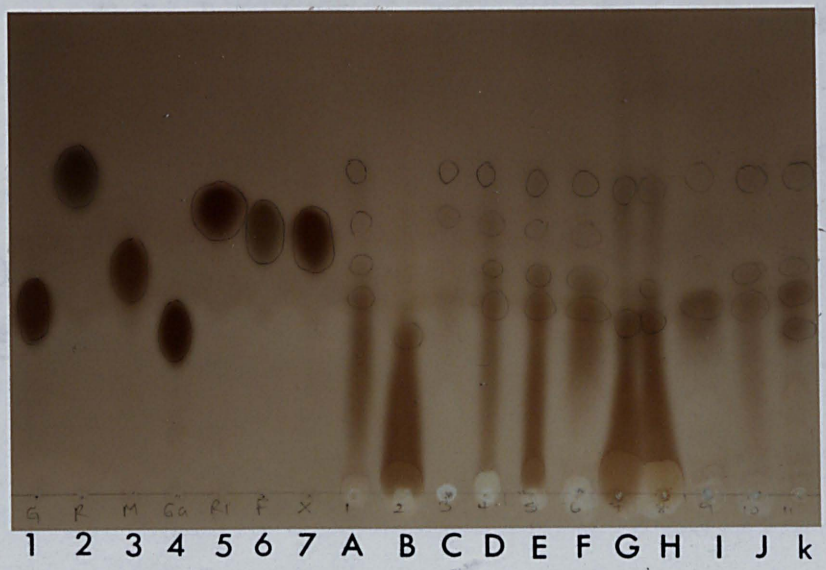


Table 6.2

Summary of the neutral sugars detected in the EPS isolated from cells grown under different nutrient limitations

Organism	Media	Neutral sugars detected by TLC
P.fragi	Carbon limited	Rhamnose, Xylose, Glucose, Galactose
	Carbon limited +YE[1]	Galactose
	Nitrogen limited	Rhamnose, Xylose, Galactose
	Nitrogen limited +YE	Rhamnose, Xylose, Glucose, Galactose
S.cohnii	Carbon limited	Rhamnose, Xylose, Glucose, Galactose
	Carbon limited +YE	Rhamnose, Xylose, Glucose, Galactose
	Nitrogen limited	Rhamnose, Galactose
	Nitrogen limited +YE	Rhamnose, Glucose, Galactose
S.liquefaciens	Carbon limited	Rhamnose, Glucose
	Carbon limited +YE	Rhamnose, Glucose, Galactose
	Nitrogen limited	*
	Nitrogen limited +YE	Rhamnose, Glucose, Galactose

[1] 0.1% (v/v)of dialysate of yeast extract was added (section 2.4.2.4)

* Below detection limit

Populations are generally thought to respond genetically to long term changes in the environment, however, if the changes are sufficiently short term and occur several times over a cells life span, physiological responses are required. It has been postulated that EPS production may be a stress response, for example, *Pseudomonas* sp S9 produces and releases EPS during complete energy and nutrient starvation (Wrangstadh *et al.*, 1986). This formation of EPS represents an energy loss to the cell unless EPS production confers an ecological advantage such as promoting attachment to solid surfaces (Costerton *et al.*, 1978; Fletcher and Marshall, 1982).

Chapter four described the attachment data obtained for the three species of bacteria in pure and mixed culture. Figure 4.7 showed that *S.liquefaciens* gave similar attachment levels grown in carbon and nitrogen excess, carbon limited or nitrogen limited media. In contrast, the polysaccharide data showed that growth in nitrogen limited media resulted in the highest levels of EPS production, this would seem to suggest that EPS does not affect the attachment ability of *S.liquefaciens*. The levels of EPS produced by *S.cohnii* and *P.fragi* were generally higher than levels produced by *S.liquefaciens*, and markedly higher in nitrogen limited culture. It is possible that EPS does not play a role in the attachment of *S.liquefaciens*, but as *S.cohnii* and *P.fragi* produce copious amounts of EPS it is more likely that EPS plays a more important role in their attachment.

Mixed culture attachment studies showed that *P.fragi* and particularly *S.cohnii* attached in higher numbers to stainless steel than would be expected from their liquid phase concentrations i.e. these two species made up a higher proportion of the surface population than the liquid phase population. This may be attributed to the high levels of EPS produced by these two species.

Bacterial exopolysaccharides are thought to enhance attachment to surfaces (Costerton *et al.*, 1981; Geesey, 1982; Rutter, 1980). Analysis of the adhesion process and of the nature of polysaccharides indirectly supports the many observations that polysaccharides can act as adhesives for cells, but little is known about this interaction (Christensen, 1989). Bar-Or and Shilo (1987) examined the extracellular flocculants produced by the benthic cyanobacteria, *Phormidium* sp strain J1 and *Anabaenopsis circularis* PCC 6720, and found that the

negatively charged polysaccharides promoted adsorption to the surfaces of clay particles. The effectiveness of these polymers at flocculating clay particles depended upon the length of the molecule and the charge density. Another example of the involvement of EPS in attachment is found in the medical field, where EPS is recognised as the major virulence factor involved in staphylococcal implant infections due to the requirement of the EPS for attachment (Christensen *et al.*, 1982).

Various mechanisms by which polymers promote adhesion have been suggested. Sutherland (1980) postulated that the extracellular polymers bridged the gap between the cell and the surface, thereby overcoming the electrical repulsion. Alternatively, the polymers may bind by chemical bonds, dipole interactions and / or hydrophobic bonding (Tadros, 1980).

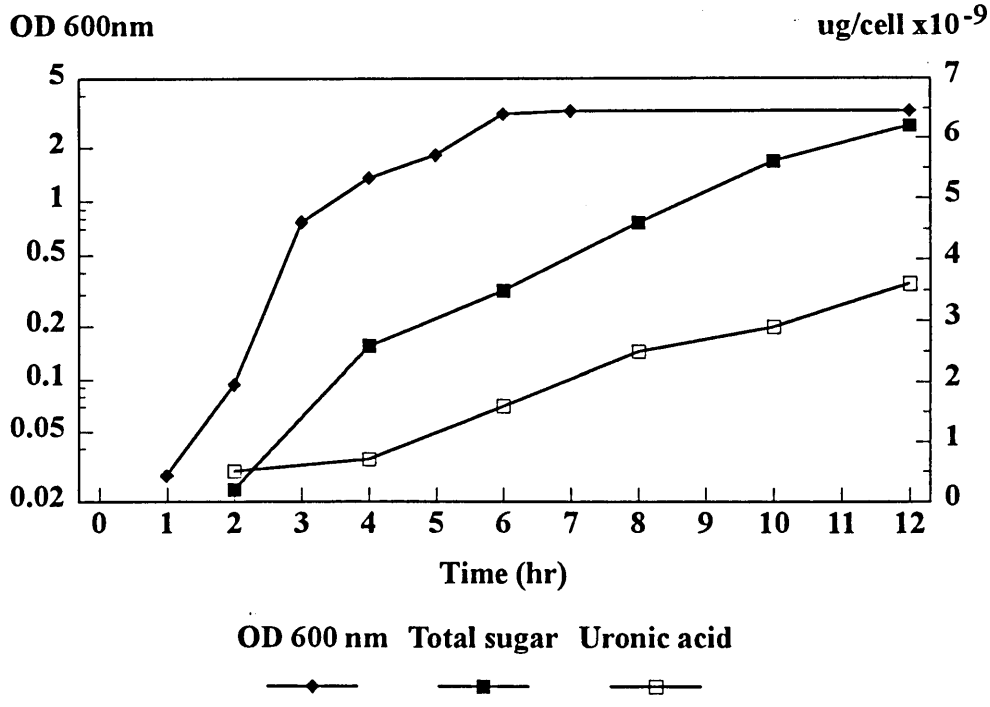
Polysaccharides, however, can also impede as well as enhance adhesion (Fattom and Shilo, 1985; Pringle and Fletcher, 1983; Rosenberg *et al.*, 1983; Wrangstadh *et al.*, 1986). Consequently general conclusions as to the role of EPS cannot be made. In the case of the bacteria in this study EPS may be involved in the attachment of *P.fragi* and *S.cohnii* but does not appear to play an important role in the attachment of *S.liquefaciens*.

6.7 Effect of growth phase on EPS production

The production of EPS through the growth cycle of *S.liquefaciens* is shown in Figure 6.15. EPS production follows the growth curve, therefore increasing during the exponential phase. However EPS continued to be synthesized well into the stationary phase at a reduced rate. As EPS was calculated per cell, the increases in total sugar and uronic acid are genuine and are not due to increases in cell numbers. The fact that EPS synthesis continued to increase in the stationary phase indicates that the polysaccharide was not used by the organism as a carbon and energy source.

Figure 6.15

Production of EPS by *S.liquefaciens* through growth in carbon and nitrogen excess media.



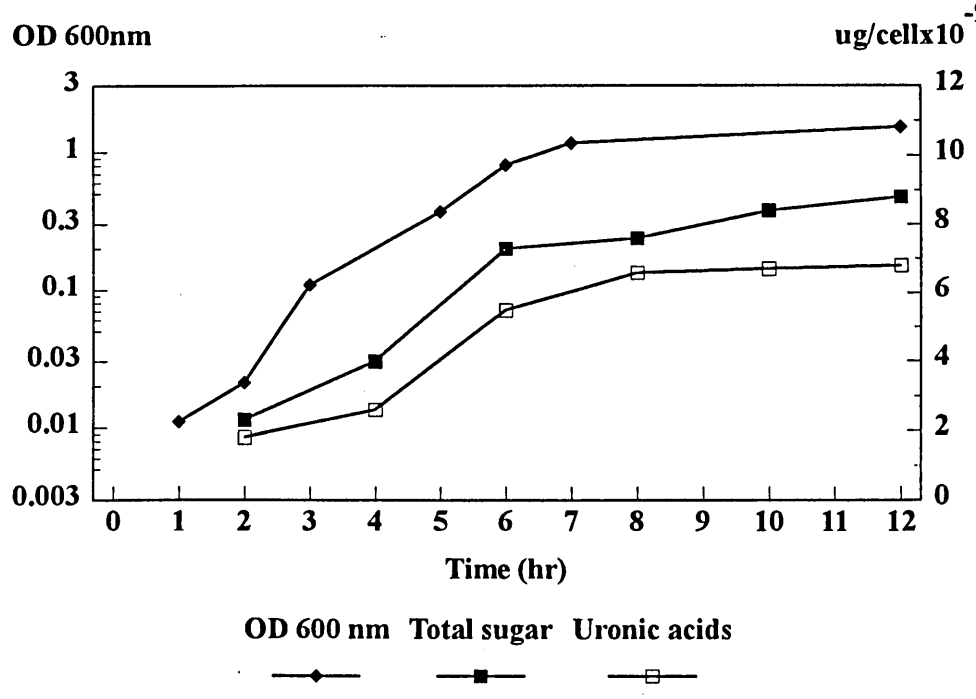
S.liquefaciens was grown in carbon and nitrogen excess medium at 25°C. Samples were removed at intervals for optical density determination and EPS isolation. EPS was quantified by total sugar and uronic acid assays.

Figure 6.16 shows that production of polysaccharide by *P.fragi* followed similar trends. Synthesis of EPS by *S.cohnii,* however, showed a slightly different pattern to the Gram negative bacteria (Figure 6.17) in that EPS production increased throughout the growth cycle and continued to increase into stationary phase at only a slightly reduced rate. The levels of uronic acids increased markedly during the stationary phase and there are two possible explanations for this. Firstly, the uronic acid components of the exopolysaccharide synthesized may be increasing with respect to the total sugar levels. Uhlinger and White (1983) found that the EPS produced by *Pseudomonas atlantica* increased with respect to uronic acid components during the exponential phase. Alternatively, another exopolysaccharide may be synthesized during the stationary phase with a high level of uronic acids. Christensen *et al.* (1985) found that the marine *Pseudomonas* sp NCMB 2021 elaborated different polysaccharides during different growth phases; one polysaccharide was produced only in the exponential phase, whereas the second polysaccharide was produced from the end of the exponential phase into the stationary phase. Pazur *et al.* (1971) also found that *Streptococcus faecalis* produced two polysaccharides in pure culture. Similarly *Rhodopeudomonas capsulata* sp11 was found to produce two different polymers (Omar *et al.*, 1983). Fletcher and Floodgate (1973) concluded the marine organism in their studies produced primary and secondary polymers.

Several authors have studies EPS production through growth with varying observations; some found EPS production to be maximal in the stationary phase, as in this study, whilst others found highest levels in the exponential phase (Table 6.3).

Figure 6.16

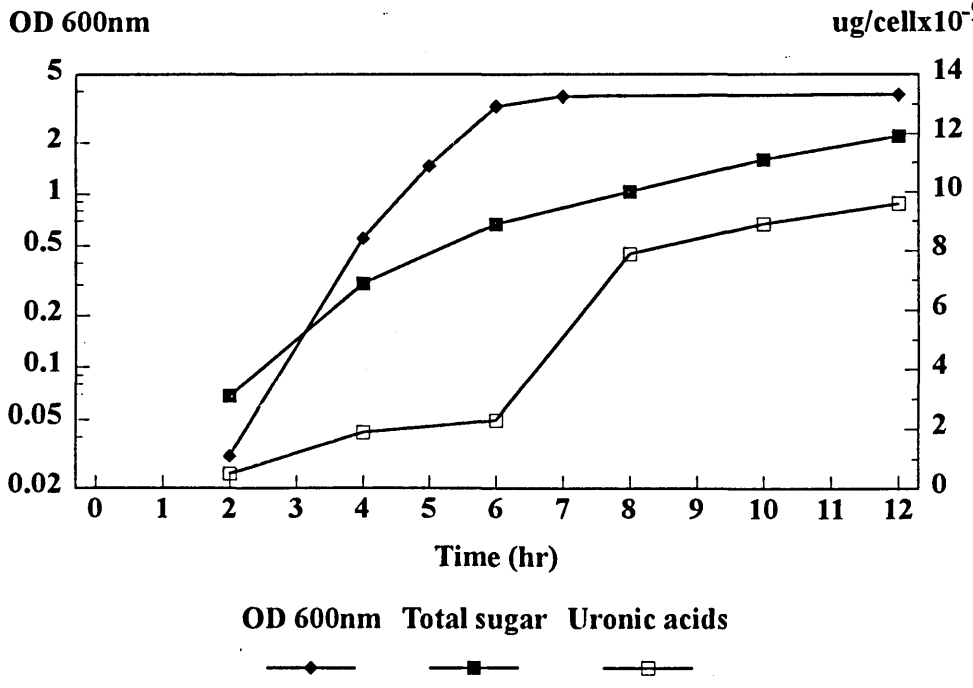
Production of EPS by *P.fragi* through growth in carbon and nitrogen excess media



P.fragi was grown in carbon and nitrogen excess medium at 25°C. Samples were removed at intervals for optical density determination and EPS isolation. EPS was quantified by total sugar and uronic acid assays.

Figure 6.17

Production of EPS by *S.cohnii* through growth in carbon and nitrogen excess media.



S.cohnii was grown in carbon and nitrogen excess medium at 25°C. Samples were removed at intervals for optical density determination and EPS isolation. EPS was quantified by total sugar and uronic acid assays.

Table 6.3

Review of the growth phases found to yield most EPS

Organism	Phase with highest EPS production	Reference
Butyrivibrio fibrisolvens nyx	Exponential	Wachenheim and Patterson, 1992
Pseudomonas putida	Stationary	Read and Costerton, 1987
Pseudomonas fluorescens	Stationary	Read and Costerton, 1987
Pseudomonas sp strain NCIB 11264	Stationary	Williams and Wimpenny, 1977
Rhizobium meliloti	Exponential	Dudman, 1964
Myxococcus xanthus	Stationary	Sutherland, 1979
Klebsiella aerogenes	Stationary	Duguid and Wilkinson, 1953
Pseudomonas atlantica	Late stationary	Uhlinger and White, 1983
Freshwater bacterium	Late exponential - early stationary	Allison and Sutherland, 1987
Pseudomonas aeruginosa	Exponential	Mian *et al.*, 1978
Haloferax mediterranei	Exponential	Anton *et al.*, 1988
Vibrio fischeri	Stationary	Rodrigues and Bhosle, 1991
Xanthomonas campestris	Late exponential - early stationary	Shu and Yang, 1990
Porphyridium sp strain UTEX637	Stationary	Arad *et al.*, 1988

The production of exopolysaccharides has been suggested as a stress response in certain organisms. Uhlinger and White (1983) found that greatest accumulation of EPS from *Pseudomonas atlantica* occurred late in the stationary phase when the cells were under maximum stress. These workers also found that the presence of uronic acids was maximal during periods of metabolic stress. Other workers have suggested that the extent of EPS production is inversely related to an organisms growth rate, and therefore particularly significant for starved cells (Robinson *et al.*, 1984). Wrangstadh *et al.* (1986) found that the marine *Pseudomonas* sp S9 produced and released an extracellular polysaccharide during complete energy and nutrient starvation. Many natural environments are characterised by nutrient fluxes and predominantly by low nutrient or stressful niches where bacteria must survive by adaptation. Consequently, the production of exopolymers may be an adaptive response to the environmental conditions. Exopolymers have been shown to protect cells from phagocytosis, bacteriophages, antibiotics, biocides, surfactants, antibodies and desiccation (Costerton *et al.*, 1985).

Fattom and Shilo (1985) suggested that the production of polymers by *Phormidium* J1 served as a dispersal mechanism in the normal life cycle which enabled the bacteria to colonize new surfaces. Rosenberg *et al.* (1983) reported a similar desorption effect for *Acinetobacter calcoaceticus* RAG1 caused by the release of a polysaccharide.

The studies on attachment through growth showed that attachment of *S.liquefaciens* was maximal during the late exponential - early stationary phase, but decreased later in the stationary phase (Figure 4.7), although EPS production continued to increase into the stationary phase. It would therefore appear that there is no relationship between EPS production and attachment of *S.liquefaciens*. It is possible that the decrease in attachment observed later in the stationary phase is due to changes in the nature of the polysaccharide, rather than related to absolute levels. There is some suggestion in the data that the composition of the exopolysaccharide of *S.cohnii* showed variable levels of uronic acids as large increases in the uronic acid levels were observed during the stationary phase.

The role of polysaccharides in attachment is somewhat controversial, promoting, inhibiting or having no effect on attachment. Polysaccharides have been reported to enhance

attachment of marine bacteria to surfaces (Costerton *et al.*, 1981; Fletcher and Floodgate, 1973; Geesey, 1982; Rutter, 1980). Marshall *et al.* (1971) concluded that the fibrous polymer produced by *Pseudomonas* sp was involved in firm adhesion. Several electron microscopy studies of attached populations have demonstrated the presence of polymeric fibrils (Fletcher and Floodgate, 1973; Zottola, 1991). Electron microscopy studies by Read and Costerton (1987) demonstrated that fibrous polysaccharide was involved in the attachment of *Pseudomonas putida* and *Pseudomonas fluorescens* and in the structural matrices of the microcolony mode of growth.

The adherence and therefore the pathogenesis of *Staphylococcus epidermidis* is dependant upon slime production (Christensen *et al.*, 1982). The adherence of *Streptococcus mutans* to smooth surfaces has also been attributed to slime production (Gibbons and Van Houte, 1980).

It is clearly an advantage for a bacterium colonizing a surface to be able to synthesize EPS whilst growing to increase the strength of the adhesive bond and to build up a protective glycocalyx (Read and Costerton, 1987). The mechanism by which the polymer produced by free living cells aids attachment may be by overcoming the electrical repulsion between the negatively charged surface and the bacterium, although EPS is negatively charged it may facilitate adsorption by hydrophobic interactions (Bengtsson, 1991).

In contrast, however, some workers have demonstrated an inverse relationship between adsorption and EPS production (Fattom and Shilo, 1985; Pringle and Fletcher, 1983; Rosenberg *et al.*, 1983). Wrangstadh *et al.* (1986) found that the presence of polysaccharide on the cell surface correlated with decreases in adhesion to hydrophobic surfaces. Paul and Jeffrey (1985) suggested it was unlikely that slimes and extracellular polymeric substances could be involved in the initial adhesion process of bacteria to substrata in the marine environment because of their common hydrophilic properties and loose connection to cells. Allison and Sutherland (1987) studied the attachment of a non polysaccharide producing mutant and the wild type to glass. They found the non polymer producing mutant attached in similar numbers to the wild type, but did not form microcolonies. These data would seem to suggest that EPS is not involved in the attachment process itself, but is required for

microcolony and biofilm development. Wardell *et al.* (1983) also concluded that EPS was not directly involved in initial attachment but in the development of the microbial film.

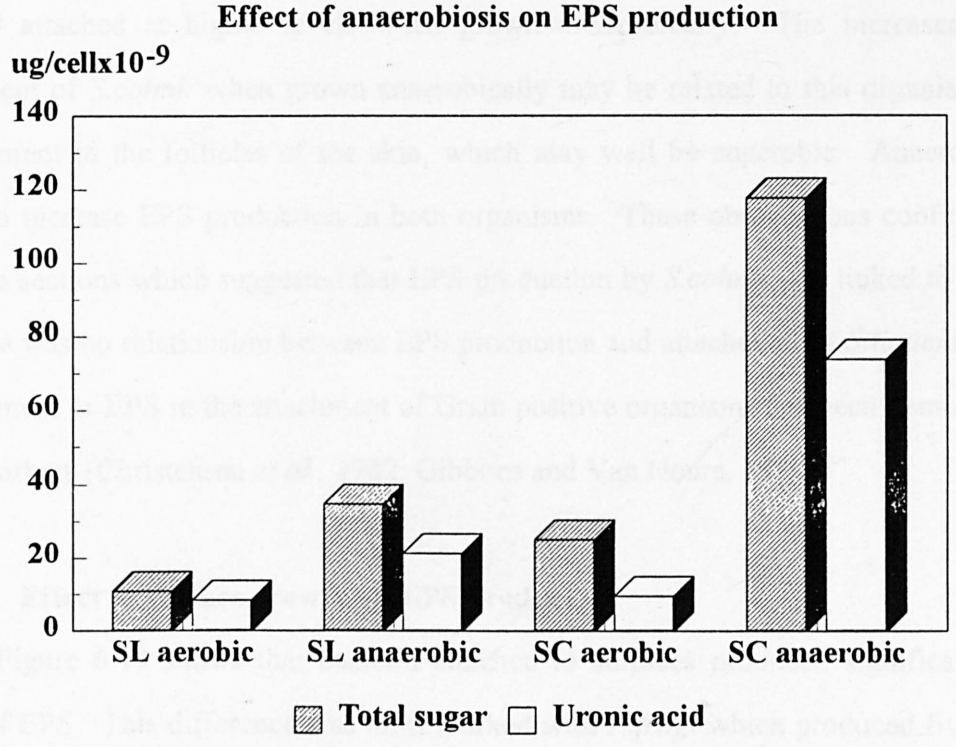
The reported effects of EPS production on attachment are varied. From the data presented here, it can be concluded that EPS does not inhibit attachment, and in the case of *S.cohnii* and *P.fragi* high levels of EPS production may relate to the increased levels of attachment observed in mixed culture. The attachment of *S.liquefaciens* appears to be independent of EPS production and therefore other cell surface characteristics such as hydrophobicity and charge may play a role.

6.8 Effect of anaerobiosis

Figure 6.18 shows that anaerobic growth results in increased levels of EPS ; a four fold increase was observed in *S.liquefaciens* and a six fold increase in *S.cohnii*. (*P.fragi* is an obligate aerobe). These observations are in contrast to those of Sutherland (1980) who found that vigorous aeration increased polysaccharide synthesis. However, if EPS synthesis is a response to environmental stresses then anaerobic conditions would be expected to produce increased levels of EPS. Hussain *et al.* (1992) found that EPS production by *Staphylococcus epidermidis* was dependant on carbon dioxide levels in the environment. In this study anaerobic conditions were produced by flushing with nitrogen which presumably also reduced the levels of other atmospheric gases. Sutherland (1985) reported that xanthan of low pyruvate content was produced at reduced aeration levels, whilst a high pyruvate polymer was produced at higher aeration levels. Alginate production by *Azotobacter vinelandii* is also dependant on the dissolved oxygen tension (Sutherland, 1985).

Why should anaerobiosis promote EPS production ? It may be a dispersal mechanism, if as suggested by some workers EPS promotes detachment or inhibits adhesion. Consequently anaerobic conditions at the surface would result in detachment of the cells to allow movement to more favourable aerobic conditions.

Figure 6.18



S.cohnii (SC) and *S.liquefaciens* (SL) were grown anaerobically as described in section for 16 hr at 25°C. EPS was precipitated using isopropanol and the EPS quantified by total sugar and uronic acid assays.

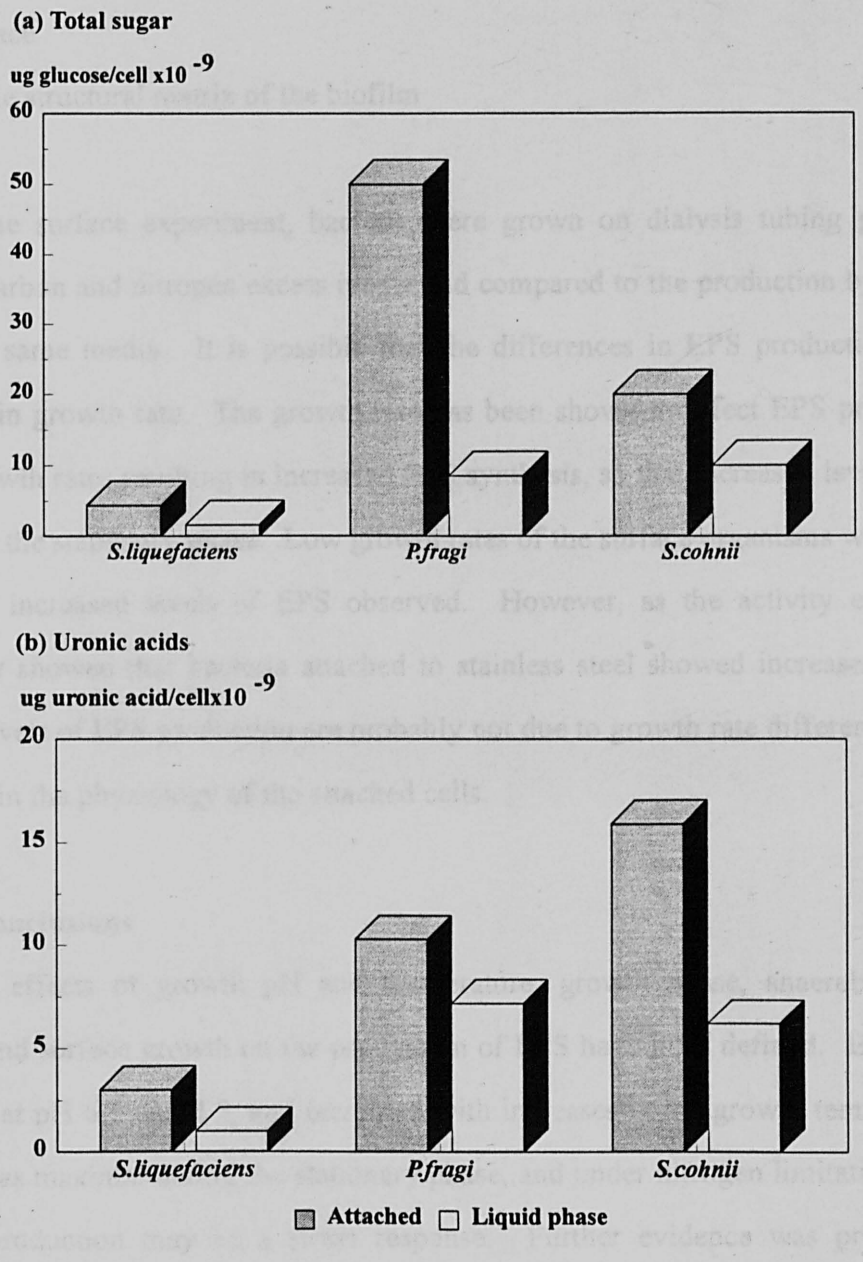
The attachment studies on aerobically and anaerobically grown cells (Figure 4.8) showed that *S.liquefaciens* attached in higher numbers when aerobically grown, whilst *S.cohnii* attached at higher levels when grown anaerobically. The increased levels of attachment of *S.cohnii* when grown anaerobically may be related to this organisms' natural environment in the follicles of the skin, which may well be anaerobic. Anaerobiosis was found to increase EPS production in both organisms. These observations confirm those in previous sections which suggested that EPS production by *S.cohnii* was linked to attachment but there was no relationship between EPS production and attachment of *S.liquefaciens*. The involvement of EPS in the attachment of Gram positive organisms has been demonstrated by other workers (Christensen *et al.*, 1982; Gibbons and Van Houte, 1980).

6.9 Effect of surface growth on EPS production

Figure 6.19 shows that bacteria attached to surfaces produced significantly higher levels of EPS. This difference was most marked with *P.fragi* which produced five times the liquid phase levels of EPS when attached to a surface. The production of EPS by surface attached bacteria has been demonstrated by various electron microscopy studies (Fletcher and Floodgate, 1973; Zottola, 1991). Uhlinger and White (1983) found that the addition of sand to cultures of *Pseudomonas atlantica* stimulated EPS production (24 fold), possibly due to increased production by adherent bacteria. Bengtsson *et al.* (1991) found that bacteria adsorbed to mineral particles produced six times as much exopolymer as free living bacteria, but the polymer composition was unchanged. Beech *et al.* (1991) examined the effect of the addition of mild steel and stainless steel to cultures of *Pseudomonas fluorescens* and *Desulfovibrio desulfuricans*. Significantly more EPS was harvested from pure and mixed cultures of *D.desulfuricans* incubated with mild steel and stainless steel coupons. In addition, the EPS isolated from free and attached cells was found to differ in chemical composition.

Figure 6.19

Effect of surface growth on EPS production



Bacteria were grown on dialysis membrane on the surface of solidified carbon and nitrogen excess media, and the EPS produced compared to that produced by liquid cultures of the same media.

Increased EPS production by surface micro-organisms may serve several roles:

(i) Protection of the attached cells from biocides and desiccation

(ii) Enhance the adhesion, by increasing the strength and number of bonds between the cell and the surface

(iii) Form the structural matrix of the biofilm

In the surface experiment, bacteria were grown on dialysis tubing placed on the surface of carbon and nitrogen excess media and compared to the production by liquid phase cells in the same media. It is possible that the differences in EPS production are due to differences in growth rate. The growth rate has been shown to affect EPS production with reduced growth rates resulting in increased EPS synthesis, so that increased levels of EPS are produced in the stationary phase. Low growth rates of the surface organisms would therefore explain the increased levels of EPS observed. However, as the activity experiments in chapter four showed that bacteria attached to stainless steel showed increased activity, the increased levels of EPS production are probably not due to growth rate differences but due to differences in the physiology of the attached cells.

6.10 Conclusions

The effects of growth pH and temperature, growth phase, anaerobiosis, nutrient limitation and surface growth on the production of EPS have been defined. EPS production was higher at pH 6.5 and 8.5, and increased with increases in the growth temperature. EPS synthesis was maximal during the stationary phase, and under nitrogen limitation, suggesting that EPS production may be a stress response. Further evidence was provided by the observation that anaerobic growth conditions also resulted in significant increases in EPS production. Finally, surface organisms were shown to produce significantly higher amounts of EPS illustrating the involvement of EPS in the surface colonization process.

CHAPTER SEVEN

7. EXAMINATION OF CELL SURFACE HYDROPHOBICITY AND CHARGE

7.1 Introduction

Bacterial attachment to surfaces and biofilm development are influenced by cell surface characteristics such as surface charge (Fletcher and Loeb, 1979), hydrophobicity (Van Loosdrecht *et al.*, 1987a) and the presence of particular surface structures such as specific proteins, flagella and fimbriae and extracellular polysaccharides (Fletcher and Floodgate, 1973). In order for bacteria to colonize a surface and yet be able to disperse to new environments, the cell surface associated properties must respond to changes in the environment (Bayer and Sloyer, 1990; Gilbert *et al.*, 1991).

The data in previous chapters demonstrated the variability of the cell envelope in terms of OMPs, LPS and EPS expression that occurred in response to the environmental growth conditions and showed that the nature of the growth environment had profound influences on the regulatory mechanisms determining surface properties of bacteria (Allison *et al.*, 1990a). In addition, cell surface hydrophobicity and charge are also subject to environmental modification as these are physico-chemical properties of the cell surface and therefore dependant upon the surface composition (Kjelleberg and Hermansson, 1984; Wrangstadh *et al.*, 1986; Van Loosdrecht *et al.*, 1987b; Bayer and Sloyer, 1990).

Bacterial hydrophobicity is a term to describe the hydrophobic properties conferred on bacterial cells by their outermost cell surfaces, and is not necessarily associated with repulsion between water and the surface as attractive interactions exist between water and hydrocarbons although these are smaller than the attractions of water itself (Van der Mei *et al.*, 1991). Bacterial cell surface hydrophobicity measurements generally allow the distinction between more or less hydrophobic strains using a variety of methods based on; low or high water contact angles on bacterial lawns (Busscher *et al.*, 1984; Van Loosdrecht *et al.*, 1987a); low or high adsorption to a hydrophobic phase such as hydrocarbons (Rosenberg *et al.*, 1980) or octyl or phenyl sepharose (Smyth *et al.*, 1978).

Particles in aqueous suspension, including bacteria acquire a surface charge by the adsorption of ions or the ionization of surface charged groups. The majority of the charge on

bacterial cell surfaces occurs due to the ionization of charged groups on the cell surface. Bacteria possess a net negative charge at neutral pH, however decreasing the pH decreases the net negative charge until the isoelectric point is reached, typically around pH 3. An electric double layer develops around the charged particle due to reorganisation of the ions in the aqueous phase. As the net charge of the cell occurs due to the ionization of surface molecules, environmental changes that result in the modification of the cell envelope may alter the surface charge e.g. polymer production by *Staphylococcus aureus* significantly alters the cell surface charge (James, 1991).

The nature and magnitude of the charge has been determined by various methods including electrophoretic mobility measurements (Van Loosdrecht *et al.*, 1987b), isoelectric focusing (Sherbert and Lakshmi, 1986) and ion exchange chromatography (Pederson, 1980).

Cell surface charge and hydrophobicity are thought to play important roles in the non specific attachment of cells to surfaces, in contrast to attachment to epithelial cells, for example, which may also involve specific receptors. The importance of hydrophobic interactions in adherence of bacteria to epithelial cells has been demonstrated for *Streptococcus salivarius* (Weerkamp *et al.*, 1987) and *Escherichia coli* (Smyth *et al.*, 1978). Similarly, Kjelleberg and Hermansson (1984) found that increased levels of hydrophobicity correlated to attachment to glass surfaces in several of the strains they examined. Van Loosdrecht *et al.* (1987b) studied the relationship between the physicochemical surface characteristics (charge and hydrophobicity) and the attachment of bacterial cells to polystyrene. These workers concluded that both characteristics played roles in attachment although their relative importance varied between organisms.

7.2 Aims

The aims of this chapter were to compare methods of analysis of hydrophobicity, to examine the effect of growth conditions on the charge and hydrophobicity of *S.liquefaciens*, *P.fragi* and *S.cohnii*, and to evaluate the relationship between these physicochemical surface characteristics and the attachment abilities exhibited by these organisms to stainless steel.

7.3 Comparison of Bacterial Adhesion to Hexadecane (BATH) and Hydrophobic Interaction Chromatography (HIC)

Table 7.1 shows the hydrophobicities of *S.liquefaciens*, *P.fragi* and *S.cohnii* grown under different conditions as measured by BATH and HIC. The HIC results show that *S.cohnii* and *P.fragi* are hydrophobic organisms and *S.liquefaciens* relatively hydrophilic. The BATH data does not show the same distinction between the hydrophobicities of the organisms. The effect of the growth media was most marked in the BATH results with limiting growth media producing an increase of the hydrophobicities of the organisms. This trend was also observed in the HIC data although the magnitude of the difference was lower. Hydrophobic bacteria have been shown to adhere to a greater extent than hydrophilic strains (Van Loodrecht *et al.*, 1987a) and therefore in a limiting environment these organisms would have an ecological advantage as they would be more likely to attach at the solid liquid interface where nutrients are thought to concentrate.

The degree of correlation between hydrophobicity assays is varied. Mozes and Rouxhet (1987) found good agreement between HIC and MATH (Microbial Adhesion to Hexadecane) for the hydrophobic strains and very the hydrophilic strains that were used, however evaluation of strains with intermediate hydrophobicity did not show any correlation. This compares favourably with the data in this study, as the correlation between the assays was better for *P.fragi* a hydrophobic organism compared to *S.liquefaciens* which was relatively hydrophilic.

HIC and BATH assess hydrophobicity in terms of adhesion to a hydrocarbon phase and therefore may be expected to give data with good correlations. However, Van der Mei *et al.* (1987) found poor correlations even for these similar assay types. In contrast, Jones *et al.* (1991) found a good correlation between BATH and HIC, although not between these techniques and others such as the Salt Aggregation Technique (Rozgonyi *et al.*, 1985).

Table 7.1

Comparison of BATH and HIC hydrophobicity assays

Organism	Growth media	% Adhesion to hexadecane	% Adhesion to phenylsepharose
S.liquefaciens	Carbon limited	21.4 (4.3)	56.3 (0.5)
	Nitrogen limited	15.6 (2.9)	55.7 (1.1)
	Carbon and nitrogen excess	28.7 (3.1)	60.4 (0.8)
	LB	19.3 (2.7)	63.7 (1.3)
S.cohnii	Carbon limited	62.8 (4.1)	98.0 (1.1)
	Nitrogen limited	42.0 (5.0)	97.2 (2.0)
	Carbon and nitrogen excess	7.57 (2.6)	94.4 (0.7)
	LB	37.2 (4.3)	95.5 (1.4)
P.fragi	Carbon limited	87.2 (3.4)	90.1 (2.1)
	Nitrogen limited	80.5 (3.0)	93.2 (0.9)
	Carbon and nitrogen excess	38.0 (2.7)	86.4 (1.6)
	LB	43.9 (5.1)	85.4 (1.5)

Figures are means of triplicate samples assayed on two separate occasions with standard deviations in brackets

HIC measures the hydrophobic interaction between the bacterial cells and non polar phenyl ligands, whilst BATH measures adhesion to hydrocarbons such as hexadecane where interfacial tensions between the water and organic phases are high (Kjelleberg, 1984). HIC is thought to be the more sensitive method for hydrophilic organisms, whilst BATH is thought to be more appropriate for hydrophobic organisms (Gilbert *et al.*, 1991). This was

demonstrated by the data in this study as the adhesion values obtained for the hydrophobic organisms (*S.cohnii* and *P.fragi*) are markedly lower as measured by the BATH technique.

One possible problem with the HIC method is the mechanical entrapment in the gel matrix, however preliminary studies with Sepharose showed that this did not occur with the size of particles used in these studies. The data shows that the BATH results were more variable as the standard deviations were higher, and therefore HIC was chosen for further studies of hydrophobicity.

7.4 Effect of nutrient limitation

Figure 7.1 shows the effect of nutrient limitation on the hydrophobicity and charge of stationary phase cultures. Growth of *P.fragi* and *S.cohnii* in carbon or nitrogen limited media resulted in increased cell surface hydrophobicity, however the hydrophobicity of *S.liquefaciens* was reduced by growth in carbon or nitrogen limited media.

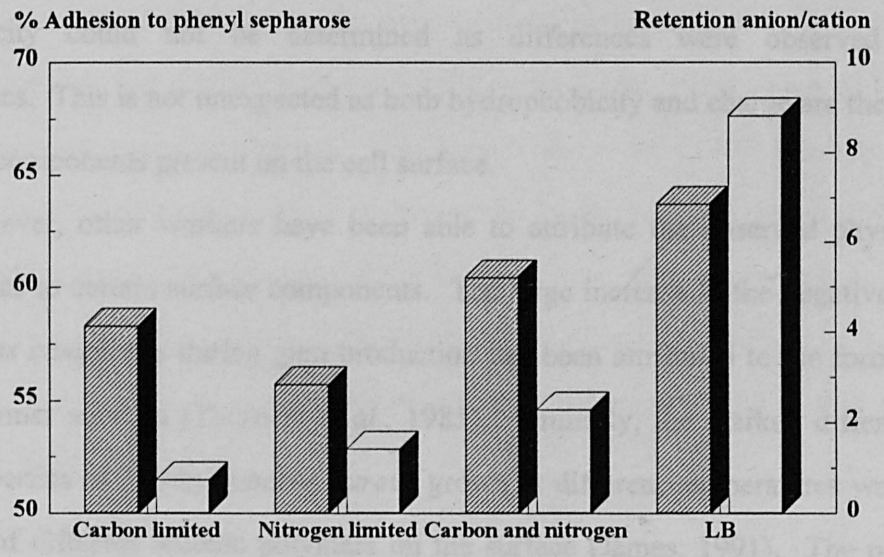
Charge was determined using electrostatic interaction chromatography where the retention by anion and cation exchange resins is measured by reading the optical density of the eluate and comparing to the original optical density (Pederson, 1980). The ratio of percentage retention by the anion to cation exchange resin was calculated, so that an increase in the ratio indicated an increase in the negative nature of the cell surface. In *S.cohnii* and *S.liquefaciens* the ratio of retention by the anion to cation exchange resin was lowest on carbon limited media and highest in organisms grown in the complex media (LB). Consequently cells grown in complex media had a greater net negative charge.

The nature of the growth environment has profound effects on the regulatory mechanisms determining the surface properties of bacteria (Allison *et al.*, 1990). The results presented in chapters five and six showed the variation in outer membrane protein, lipopolysaccharide and extracellular polysaccharide expression with growth conditions. Polysaccharide capsules as well as portions of the LPS molecule are negatively charged at neutral pH, and therefore changes in these surface molecules may affect the magnitude of the net surface charge (Bayer and Sloyer, 1990). Hydrophobicity of the cell surface is also

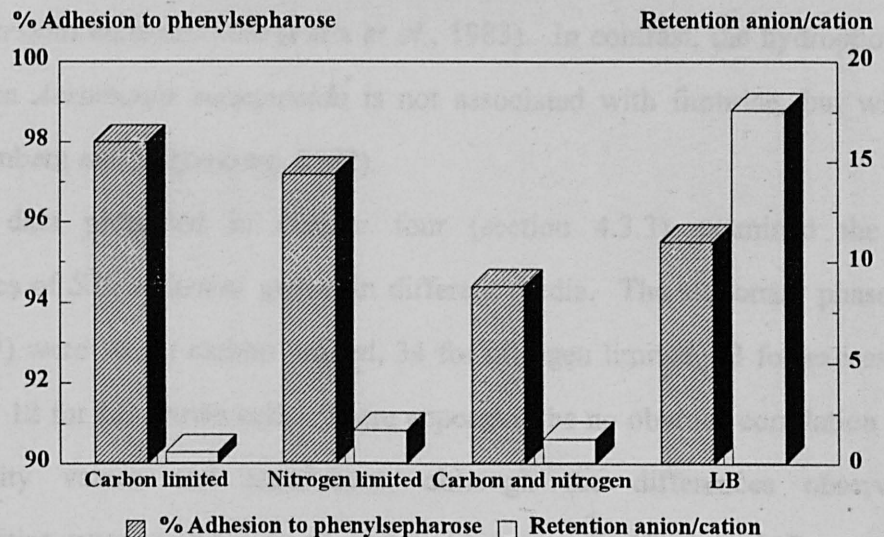
Figure 7.1

Effect of nutrient limitation on the hydrophobic and charge properties of (a) *S.liquefaciens* and (b) *S.cohnii*

(a) *S.liquefaciens*



(b) *S.cohnii*



dependent on the surface composition with induction of a surface protein in *Aeromonas salmonicida,* for example, increasing cell surface hydrophobicity (Parker and Munn, 1984).

The changes that were observed in cell surface hydrophobicity and charge must be due to the differences observed in outer membrane proteins, lipopolysaccharides and in the levels and nature of the exopolysaccharide synthesized. A single determinant of charge or hydrophobicity could not be determined as differences were observed in several characteristics. This is not unexpected as both hydrophobicity and charge are the net result of the various components present on the cell surface.

However, other workers have been able to attribute the observed physicochemical characteristics to certain surface components. The large increase in the negative mobility of *Xanthomonas campestris* during gum production has been attributed to the formation of the anionic polymer xanthan (Thornart *et al.*, 1985). Similarly, the marked differences in the surface properties of *Staphylococcus aureus* grown at different temperatures was due to the production of different anionic polymers on the surface (James, 1991). The production of many types of fimbriae is associated with high surface hydrophobicity in several species including *Yersinia enterocolitica* (Faris *et al.*, 1983). In contrast, the hydrophobicity of the fish pathogen *Aeromonas salmonicida* is not associated with fimbriae, but with envelope protein (Evenberg and Lugtenberg, 1982).

The data presented in chapter four (section 4.3.3) examined the attachment characteristics of *S.liquefaciens* grown in different media. The stationary phase attachment ratios ($x10^{-3}$) were 16 for carbon limited, 34 for nitrogen limited, 23 for excess carbon and nitrogen and 12 for LB grown cells. There appears to be no obvious correlation between the hydrophobicity values and attachment, although the differences observed in the hydrophobicities were perhaps small and unlikely to significantly influence attachment. However, the net charge of the cell surface may play a role in the attachment of *S.liquefaciens* to stainless steel, as the lowest level of attachment was observed in LB cells which had the highest ratio of retention by anion to cation exchange resins and therefore the highest net negative charge. Stainless steel also possesses a net negative charge in aqueous systems and

therefore highest levels of attachment would be expected with cells of lowest net negative charge.

Adhesion of bacteria is generally thought to be mediated by a complex interplay between hydrophobic and charge properties (Mozes *et al.*, 1987). The interactions between hydrophobic molecules on the surfaces are thought to contribute to the attractive forces, whereas repulsive forces are due to the net negative charge on the surfaces (Jones *et al.*, 1991). It is possible that this is the reason for the reduced attachment observed by LB grown cells with high net negative charges. Van Loosdrecht *et al.* (1987b) concluded that the adhesion of hydrophobic organisms is dominated by hydrophobicity, irrespective of surface charge, but the attachment of hydrophilic bacteria was primarily dependent upon the electrokinetic potential or charge. *S.liquefaciens* is a relatively hydrophilic organism and therefore the involvement of cell surface charge in its adhesion would follow the theory of Van Loosdrecht *et al.* (1987b).

7.5 Effect of anaerobiosis

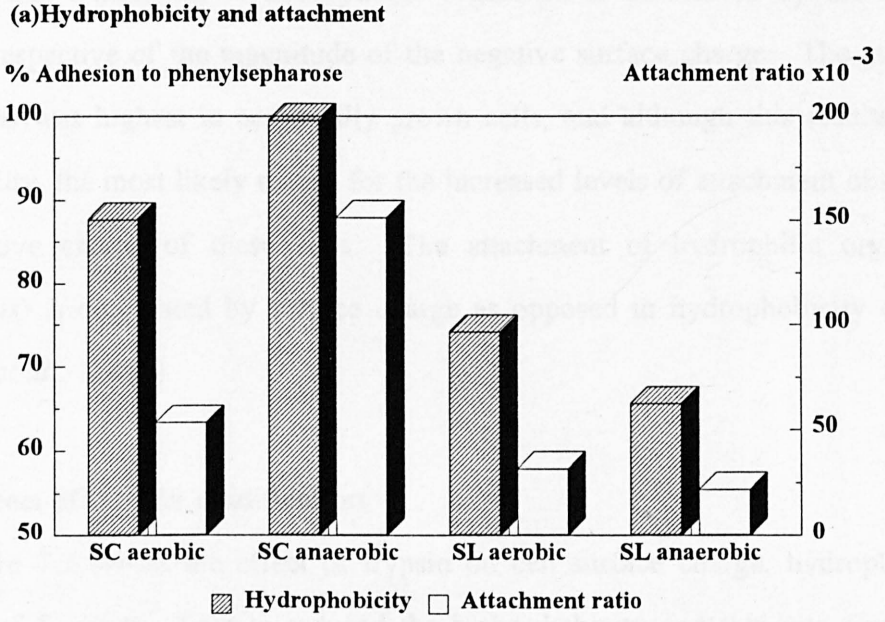
Figure 7.2 shows that anaerobic growth had differing effects on cell surface hydrophobicity. In *S.cohnii*, anaerobic growth resulted in an increase in cell surface hydrophobicity whilst *S.liquefaciens* became more hydrophilic under anaerobic conditions. It is interesting to note that these changes appeared to correlate directly with the attachment of the organisms.

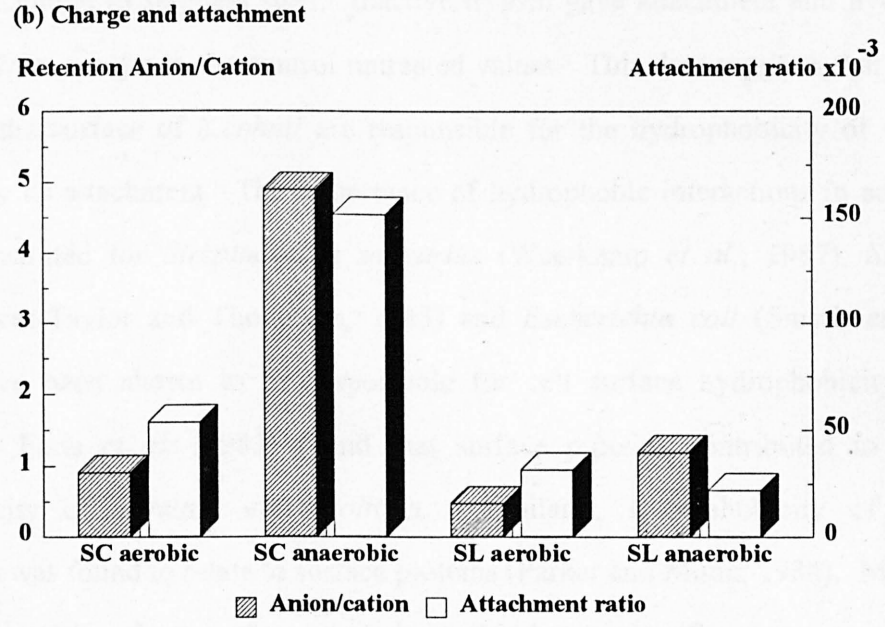
Anaerobiosis also markedly affected the charge of the cells. The results show that anaerobic growth increased the anion:cation ratio indicating an increase in the net negative charge of the cells. The charge of *S.cohnii* was most markedly affected by anaerobic growth and had a higher anion:cation ratio than *S.liquefaciens*.

Chapter five examined the changes in outer membrane proteins and lipopolysaccharides occurring with different growth conditions. Anaerobic growth induced the production of a 32kDa protein in *S.liquefaciens* and major changes were observed in the LPS profile (section 5.8). Exopolysaccharide production in both *S.cohnii* and *S.liquefaciens* was significantly higher in anaerobic cultures (section 6.8). The production of anionic polysaccharides results in an increase in the net negative charge and is thought to have a larger influence on surface charge than the charge due to LPS molecules or surface associated proteins (Bayer and Sloyer, 1990). The data shows that an increase in the net negative charge was associated with increased EPS synthesis. Thornart *et al.* (1985) reported a large increase in the negative mobility of *Xanthomonas campestris* due to anionic xanthan production and similarly the difference in surface character of *Staphylococcus aureus* was attributed to anionic polymer production (James, 1991).

Figure 7.2

Effect of anaerobiosis on (a) hydrophobicity and (b) charge of *S.liquefaciens* and *S.cohnii*





SC - *S.cohnii*, SL - *S.liquefaciens*

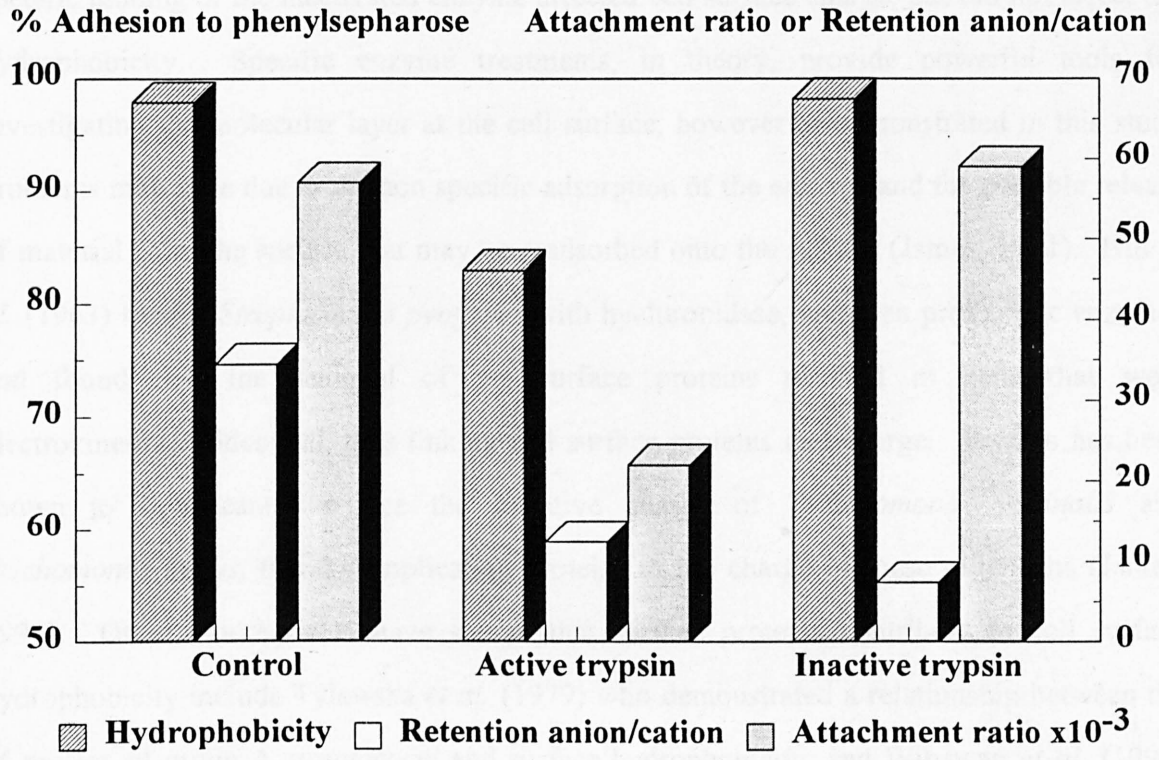
The attachment characteristics appeared to be correlated with increased cell surface hydrophobicity, however the attachment of *S.cohnii* under anaerobic growth conditions occured despite increased net negative surface charge. This would confirm the previous observation that attachment of hydrophobic organisms is dominated by the hydrophobic character irrespective of the magnitude of the negative surface charge. The attachment of *S.liquefaciens* was highest in aerobically grown cells, and although this resulted in higher hydrophobicity, the most likely reason for the increased levels of attachment observed is the lower negative charge of these cells. The attachment of hydrophilic organisms (i.e. *S.liquefaciens*) is dominated by surface charge as opposed to hydrophobicity effects (Van Loosdrecht *et al.*, 1987b).

7.6 Effect of trypsin modification

Figure 7.3 shows the effect of trypsin on cell surface charge, hydrophobicity and attachment of *S.cohnii*. Trypsin reduced the hydrophobicity and this was associated with reduced attachment to stainless steel. Inactive trypsin gave attachment and hydrophobicity results that were similar to the control untreated values. This data suggests that the proteins exposed at the surface of *S.cohnii* are responsible for the hydrophobicity of the cell and consequently its attachment. The importance of hydrophobic interactions in adherence has been demonstrated for *Streptococcus salivarius* (Weerkamp *et al.*, 1987), *Streptococcus sanguis* (Fives-Taylor and Thompson, 1985) and *Escherichia coli* (Smyth *et al.*, 1978). Proteins have been shown to be responsible for cell surface hydrophobicity in several organisms. Faris *et al.* (1983) found that surface proteins contributed to the surface hydrophobicity of *Yersinia enterocolitica.* Similarly, hydrophobicity of *Aeromonas salmonicida* was found to relate to surface proteins (Parker and Munn, 1984). McBride *et al.* (1984) also used trypsin to evaluate the relationship between surface protein expression and hydrophobicity. These workers found that the hydrophobicity of *Streptococcus mutans* was due to high molecular weight proteins which were trypsin sensitive, and modification of these proteins resulted in reduced hydrophobicity. Smit *et al.* (1992) also found that the

Figure 7.3

Effect of trypsin modification of the cell on the surface charge and hydrophobicity of *S.cohnii*



hydrophobicity and associated flocculation ability of *Saccharomyces cerevisiae* was due to proteinaceous cell surface factors.

Figure 7.3 shows that trypsin reduces the anion:cation retention ratio as compared to the control, however inactive trypsin reduces this ratio further. This suggests that non specific binding of the inactivated enzyme affected cell surface charge, but did not affect the hydrophobicity. Specific enzyme treatments, in theory, provide powerful tools for investigating the molecular layer at the cell surface, however as demonstrated in this study problems may arise due to the non specific adsorption of the enzyme and the possible release of material from the surface that may be readsorbed onto the surface (James, 1991). Hill *et al.* (1963) treated *Streptococcus pyogenes* with hyaluronidase, and then proteolytic enzymes and found that the removal of the surface proteins resulted in cells that were electrokinetically identical, thus linking cell surface proteins and charge. Trypsin has been shown to significantly reduce the negative charge of *Tritichomonas vaginalis* and *Trichomonas foetus,* thereby implicating proteins in the charge of these organisms (James, 1991). Other workers that have shown that surface proteins contribute to cell surface hydrophobicity include Tylewska *et al.* (1979) who demonstrated a relationship between the M protein of group A *streptococci* and surface hydrophobicity, and Wibawan *et al.* (1992) who showed that the hydrophobic nature of group B *streptococci* was due to surface proteins.

Evaluation of the attachment characteristics of trypsin treated *S.cohnii* in the light of the hydrophobicity and charge data indicates a direct correlation between attachment and hydrophobicity irrespective of the charge of the cells. The attachment of hydrophobic organisms such as *S.cohnii* is thought to be dominated by hydrophobicity irrespective of surface charge, whereas the attachment of hydrophilic organisms is thought to be more dependent on surface charge (Van Loosdrecht *et al.*, 1987b). In conclusion, the hydrophobicity of *S.cohnii* appears to be due (at least partially) to trypsin-sensitive surface proteins and affects the attachment of the organism to stainless steel.

Figure 7.4 shows the effect of trypsin on the hydrophobicity, surface charge and attachment of *S.liquefaciens*. Trypsin did not have a significant effect on the hydrophobicity of *S.liquefaciens*, although inactive trypsin produced a slight reduction in the hydrophobicity. This must be due to non specific adsorption of the inactivated enzyme to the cell surface, and was demonstrated in the SDS-PAGE in section 5.7. This section also showed that trypsin did not affect the outer membrane proteins of *S.liquefaciens* suggesting that these proteins were not 'visible' at the cell surface, and consequently these proteins must be unable to contribute to cell surface hydrophobicity and charge.

The anion:cation retention ratio was significantly increased by active and inactive trypsin, however as there were no detectable changes in the proteins of the outer membrane, it seems most likely that the increase in charge is due to non specific adsorption of the enzyme to the cell surface thereby influencing the net charge of the cell.

Active and inactive trypsin resulted in reduced attachment of *S.liquefaciens* (Figure 7.4), and as hydrophobicity was relatively unaffected whilst cell surface charge was significantly affected by the presence of the enzyme (albeit due to non specific adsorption), it appears that the reduced attachment observed was due to the increase in the negative charge of the cells. Adhesion of microorganisms is generally thought to be mediated by a complicated interplay of hydrophobic and charge properties (Mozes *et al.*, 1987). Interactions between hydrophobic molecules are thought to contribute to attractive forces, whereas repulsive forces are due to the net negative charges of the surfaces involved. Consequently, adsorption of the enzyme to the cell surface increased cell surface charge, thereby increasing repulsive forces between the bacterial cell surface and the stainless steel resulting in decreased levels of attachment. As previously mentioned, the attachment of hydrophilic organisms such as *S.liquefaciens* is thought to be mediated by charge as well as hydrophobicity, whilst the attachment of hydrophobic organisms is dominated by the hydrophobic character.

Figure 7.4

Effect of trypsin modification of the cell on the surface charge and hydrophobicity of *S.liquefaciens*

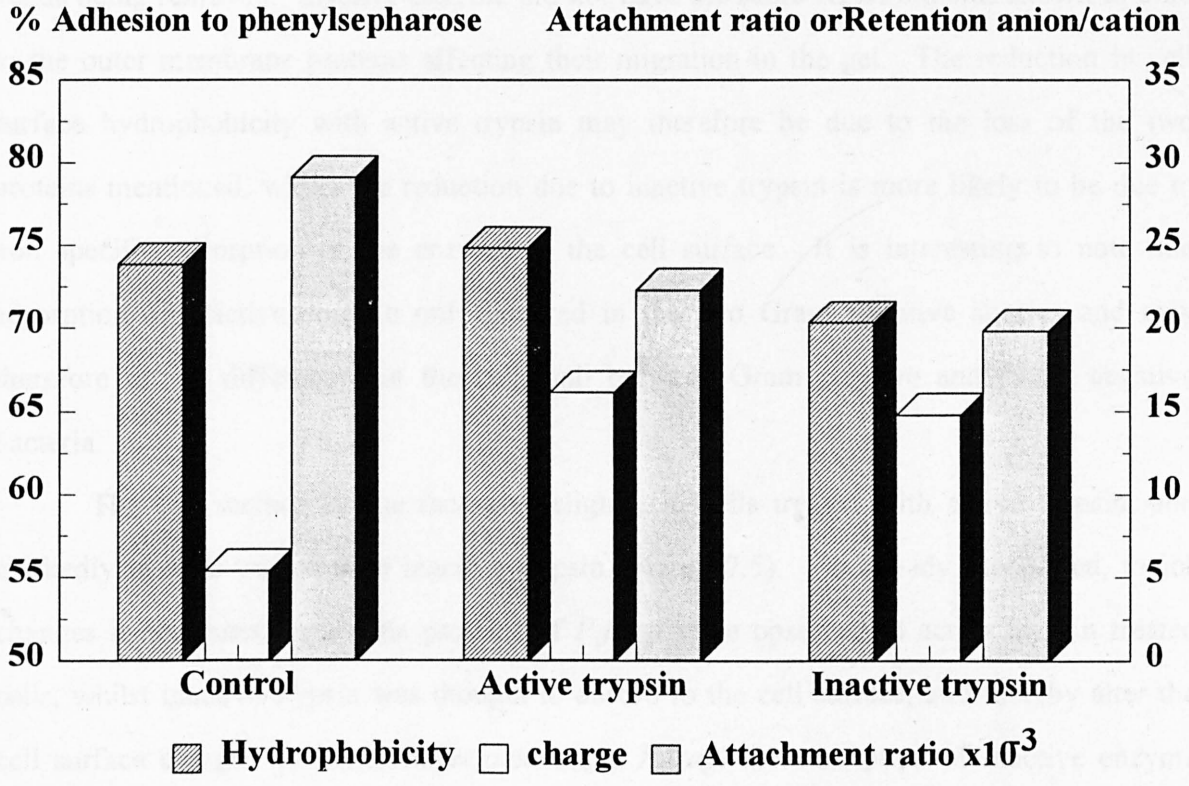


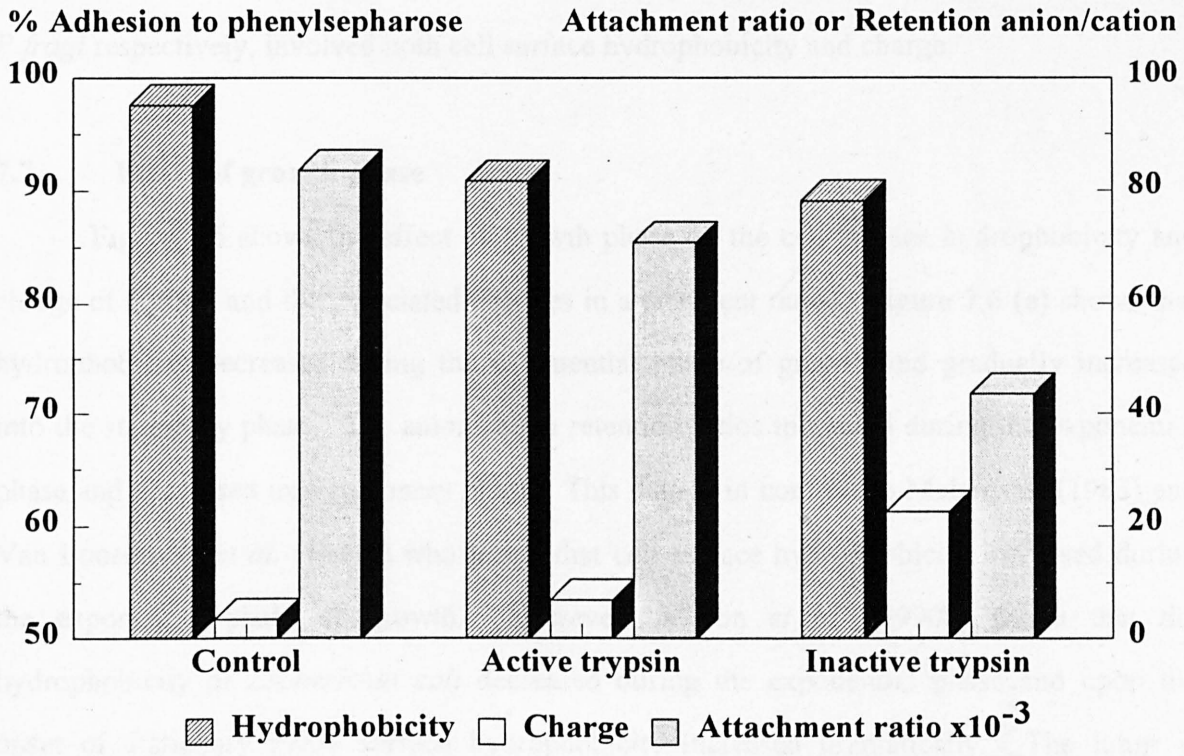
Figure 7.5 shows that active and inactive trypsin reduced the cell surface hydrophobicity of *P.fragi* to a similar extent. Section 5.7 examined the protein changes that occured due to the treatment of whole cells of *P.fragi* with trypsin and showed that the active enzyme significantly affected the outer membrane protein profile with two major protein bands being removed. Inactive enzyme did not have the same effect but was shown to bind to the outer membrane proteins affecting their migration in the gel. The reduction in cell surface hydrophobicity with active trypsin may therefore be due to the loss of the two proteins mentioned, whilst the reduction due to inactive trypsin is more likely to be due to non specific adsorption of the enzyme to the cell surface. It is interesting to note that adsorption of inactive enzyme only occured in the two Gram negative species and may therefore reflect differences in the cell wall between Gram positive and Gram negative bacteria.

The cell surface charge increased slightly in cells treated with active trypsin, and markedly in cells treated with inactive trypsin (Figure 7.5). As already mentioned, major changes in the outer membrane proteins of *P.fragi* were observed in active trypsin treated cells, whilst inactive trypsin was thought to adsorb to the cell surface, and thereby alter the cell surface charge. In both *S.liquefaciens* and *P.fragi* the adsorption of inactive enzyme resulted in increases in the anion:cation ratio.

Evaluation of the relationship between the physico-chemical properties and the attachment of *P.fragi* suggests that both hydrophobicity and charge play a role in attachment. Attachment was highest when hydrophobicity was high and the anion:cation ratio was low, and a decrease in the hydrophobicity with active trypsin was associated with an increase in the anion:cation ratio and a decrease in the level of attachment. Hydrophobic interactions are thought to contribute to the attractive forces whilst the net negative surface charge is thought to result in repulsive forces between the cell and the surface, consequently increases in hydrophobicity and decreases in cell surface charge would promote attachment. Although inactive trypsin did not significantly affect the cell hydrophobicity of *P.fragi*, the significant increase in anion:cation ratio and therefore net negative cell surface charge resulted in a

Figure 7.5

Effect of trypsin modification of the cell on the surface charge and hydrophobicity of *P.fragi.*



marked reduction in the attachment observed and therefore demonstrates the role of charge in the attachment of this organism.

In conclusion, the attachment of the most hydrophobic organism *S.cohnii* appeared to be dependent upon cell surface hydrophobicity regardless of the cell surface charge, whilst the attachment of the hydrophilic and slightly less hydrophobic organisms, *S.liquefaciens* and *P.fragi* respectively, involved both cell surface hydrophobicity and charge.

7.7 Effect of growth phase

Figure 7.6 shows the effect of growth phase on the cell surface hydrophobicity and charge of *P.fragi* and the associated changes in attachment ratios. Figure 7.6 (a) shows that hydrophobicity decreased during the exponential phase of growth and gradually increased into the stationary phase. The anion:cation retention ratios increased during the exponential phase and decreased into stationary phase. This data is in contrast to Malmqvist (1983) and Van Loosdrecht *et al.* (1987a) who found that cell surface hydrophobicity increased during the exponential phase of growth. However, Allison *et al.* (1990b) found that the hydrophobicity of *Escherichia coli* decreased during the exponential phase and upon the onset of stationary phase surface hydrophobicity increased dramatically. The latter is therefore similar to the observations described in this study.

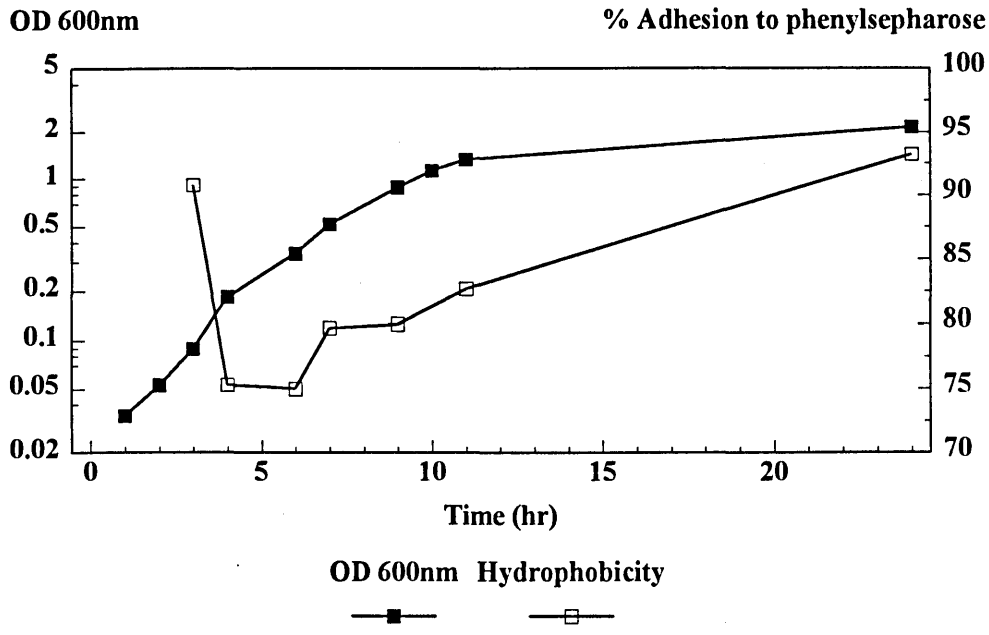
Bayer and Sloyer (1990) found no difference in the charge of *E.coli* between exponential and stationary phase cells, although Moyer (1936) had found that the electrophoretic mobility of *E.coli* decreased during the exponential phase, that is became more negative, as was observed in this study.

The attachment of *P.fragi* decreased during the exponential phase and therefore appeared to correlate with a decrease in hydrophobicity and increase in cell surface charge. Gilbert *et al.* (1991) examined the change in the physicochemical characteristics of *Staphylococcus epidermidis* and *E.coli* through growth and the attachment of the organisms to glass. As with *P.fragi*, hydrophobicity and the electronegative character of *E.coli* decreased during the exponential phase and at the same time the number of cells attaching to the glass increased. The data presented for *P.fragi* therefore agrees with the other previous

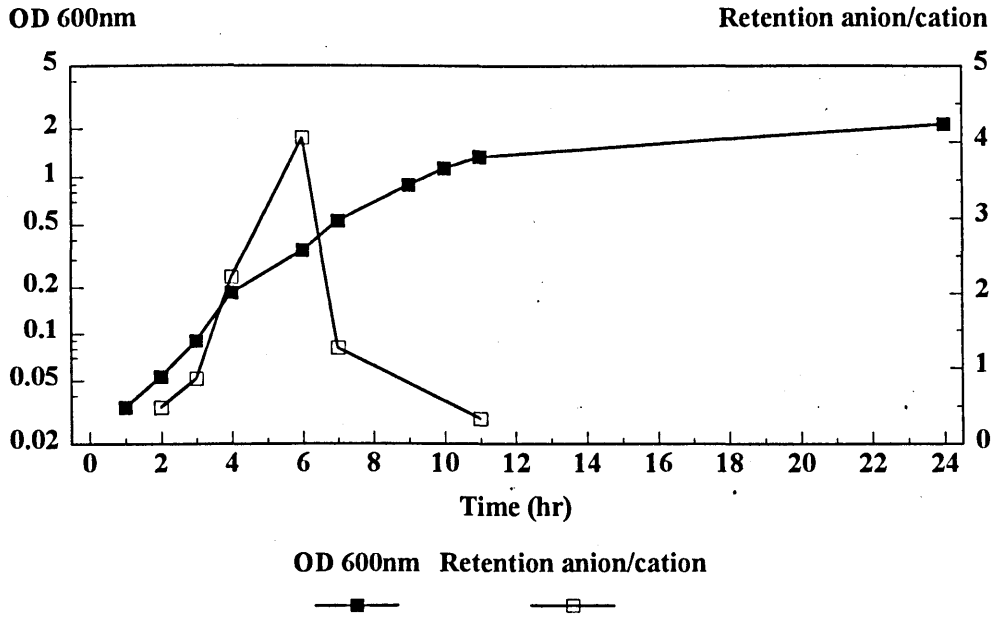
Figure 7.6

Effect of growth phase on (a) hydrophobicity, (b) charge and (c) attachment of *P.fragi*

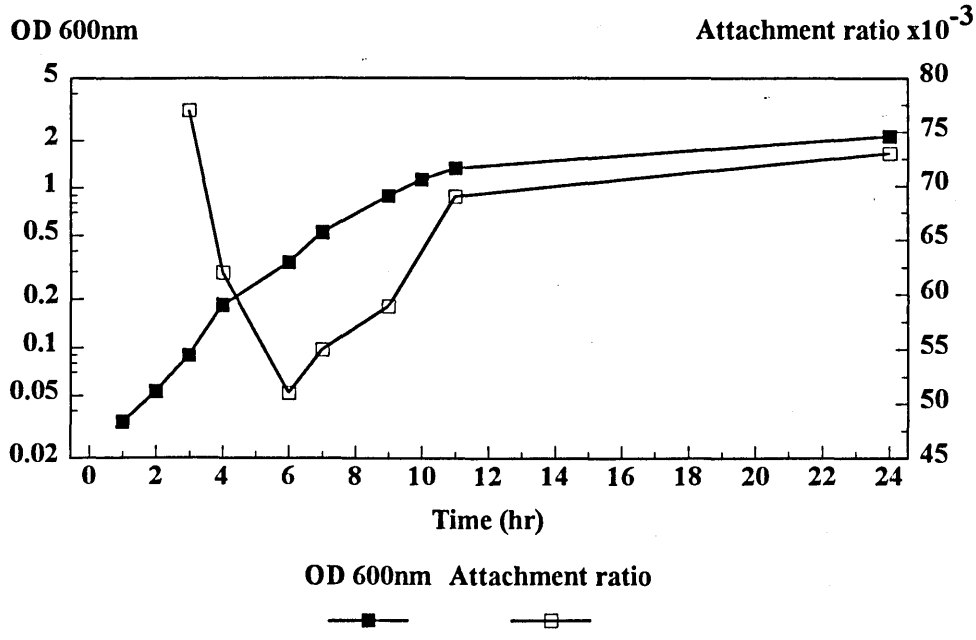
(a) Hydrophobicity



(b) Charge



(c) Attachment



studies and suggests that the attachment of *P.fragi* to stainless steel is mediated by both hydrophobic and surface charge properties.

The increases in charge observed for *P.fragi* with increased hydrophobicity appear contradictory. However, polar groups which possess high charge may only occupy a relatively small fraction of the cell surface.

Vanhaecke *et al.* (1990) examined the adhesion kinetics of various isolates of *Pseudomonas aeruginosa* to 304 and 316L stainless steel. These workers concluded that cell surface hydrophobicity was the major parameter influencing the adhesion rate and that cell surface charge was of minor importance. This is perhaps not contradictory to the data presented in this study as the changes observed in the anion:cation retention ratio were relatively small and therefore perhaps the most significant mediator of adhesion was in fact hydrophobicity.

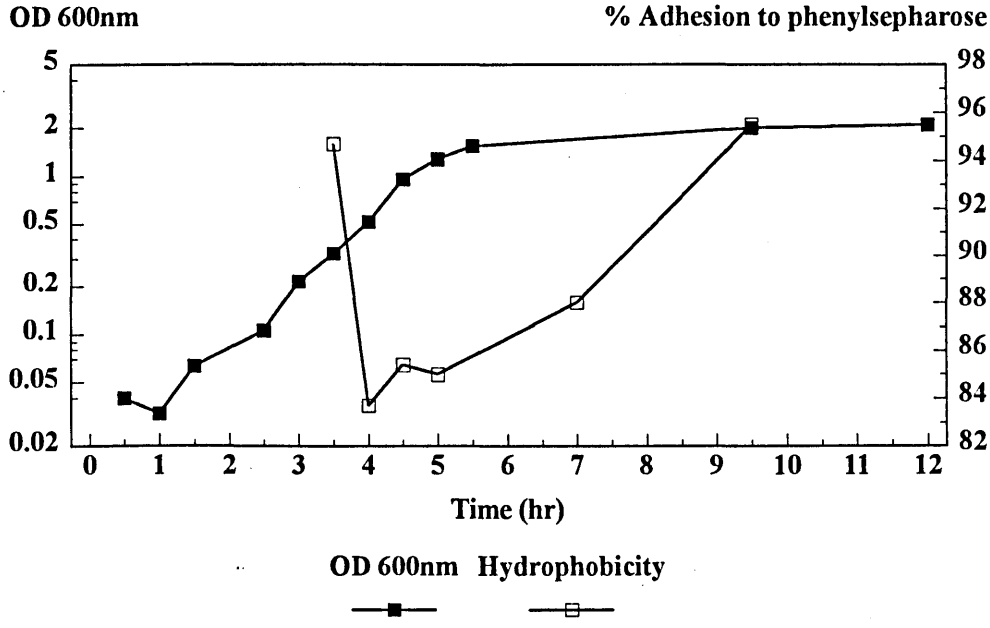
The decrease in adhesiveness during the exponential phase of growth may allow cells that are attached to a surface to more readily disperse from that surface and colonize new environments. The cell envelope shows considerable variation including its hydrophobic and charge properties, and it may be through changes in these properties due to differences in the envelope composition that allow bacteria to disperse to new environments.

Figure 7.7 shows the effect of growth phase on the surface hydrophobicity, charge and attachment of *S.cohnii*. Similar trends were observed to those for *P.fragi*. Hydrophobicity decreased during the exponential phase, increasing to the original level during stationary phase. The anion:cation retention ratio (i.e. the negative character) increased during the exponential phase and decreased during the stationary phase. The attachment levels of *S.cohnii* mirrored the hydrophobicity data, decreasing during the exponential phase. Similar observations were reported by Gilbert *et al.* (1991) for *Staphylococcus epidermidis*, although these workers found a decrease in the electronegativity of the cell during the exponential phase.

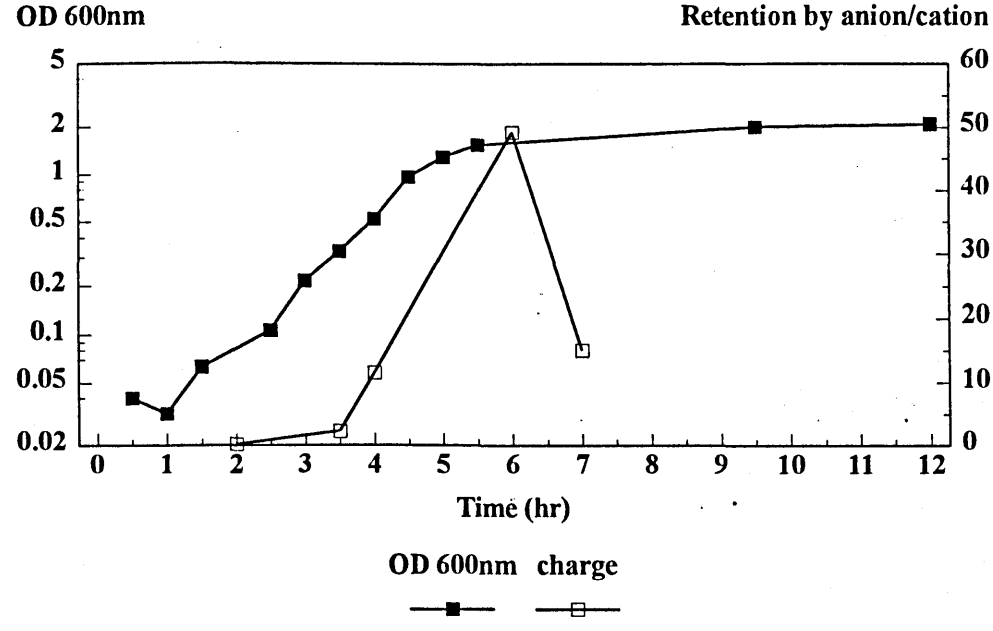
Figure 7.7

Effect of growth phase on (a) hydrophobicity, (b) charge and (c) attachment of *S.cohnii*

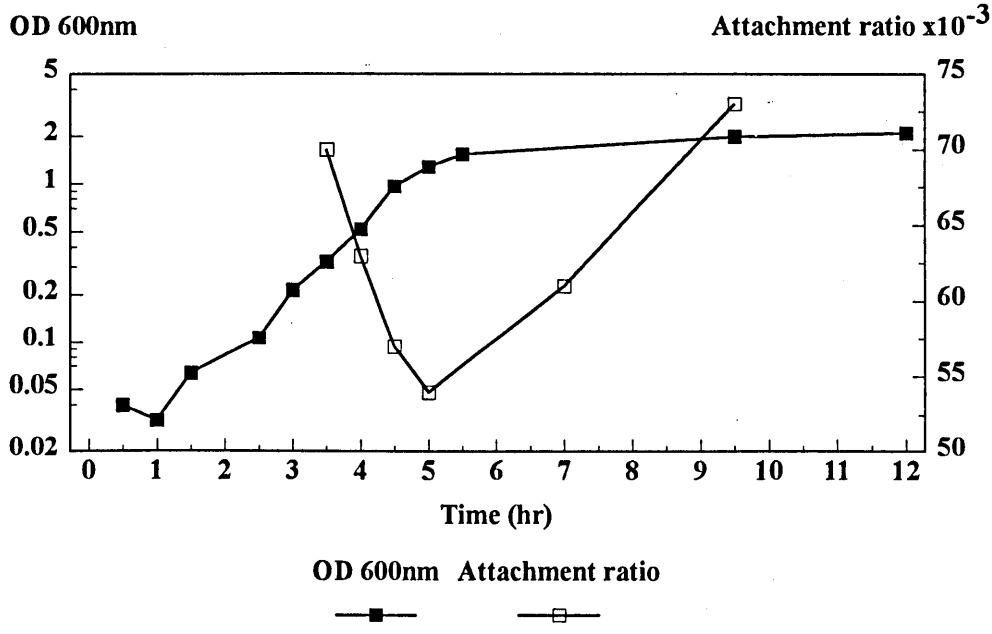
(a) Hydrophobicity



(b) Charge



(c) Attachment



S.cohnii is a highly hydrophobic organism and the attachment of hydrophobic organisms is thought to be dominated by hydrophobicity, irrespective of cell surface charge. Although this appeared to be the case in the experiments described previously, this data shows that the associated changes in cell surface charge were such as to promote adhesion. This does not disagree with the theory of Van Loosdrecht *et al.* (1987b), as surface charge in this case could have had additional beneficial effects on attachment but not playing the primary role.

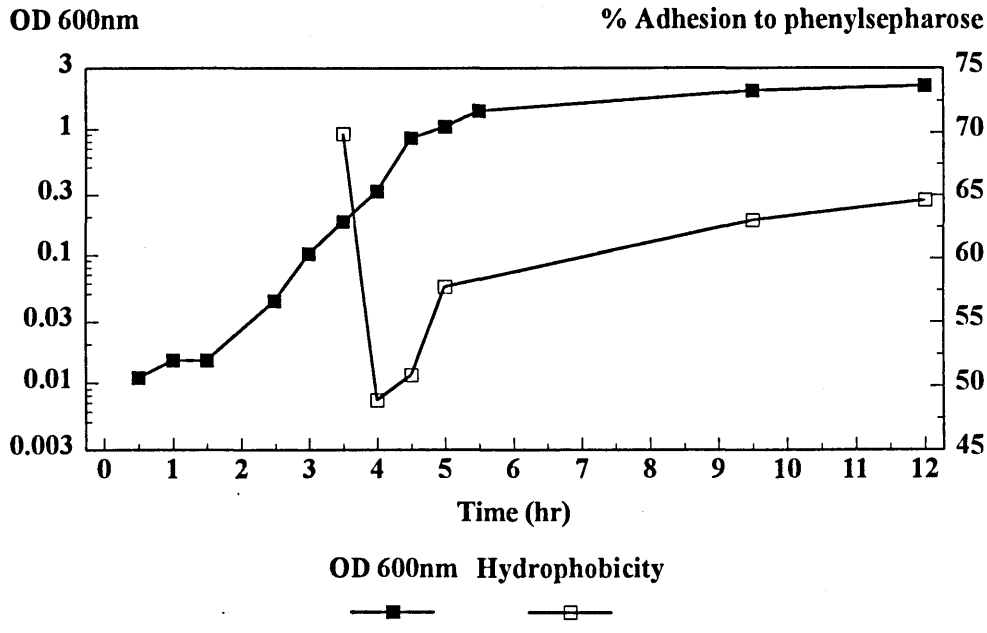
Figure 7.8 shows the effect of growth phase on the hydrophobicity, charge, cell volume and attachment of *S.liquefaciens*. As for *P.fragi* and *S.cohnii*, hydrophobicity decreased rapidly during the exponential phase and increased into stationary phase. However, in *S.liquefaciens* the anion:cation retention ratio decreased during the exponential phase, that is the cells effectively became less negatively charged. This was associated in an increase in the attachment of *S.liquefaciens* during the exponential phase of growth. The significantly different results seem to suggest that in contrast to *S.cohnii* and *P.fragi*, the attachment of *S.liquefaciens* is dominated by cell surface charge as despite decreased hydrophobicity during the exponential phase (which is normally associated with decreased attachment) attachment increased as the net negative cell surface charge decreased thereby reducing the repulsive forces between the cell surface and the stainless steel. As previously described, Van Loosdrecht *et al.* (1987b) suggested that the attachment of hydrophilic organisms such as *S.liquefaciens* is dominated by surface charge, which appeared to be the case.

It is possible that the differences in hydrophobicity and charge during the exponential phase are due to cell cycle mediated events (Allison *et al.*, 1990b: Gilbert *et al.*, 1991). Allison *et al.* (1990b) achieved growth rate control of an adherent population of *E.coli* by growing the population on a perfused filter system and this enabled them to collect the newly shed daughter cells. These workers found that the daughter cells were more hydrophilic than the planktonic or biofilm cells and concluded that hydrophobicity changed during the cell division cycle, and was low at and immediately after division of *E.coli*. It was suggested that

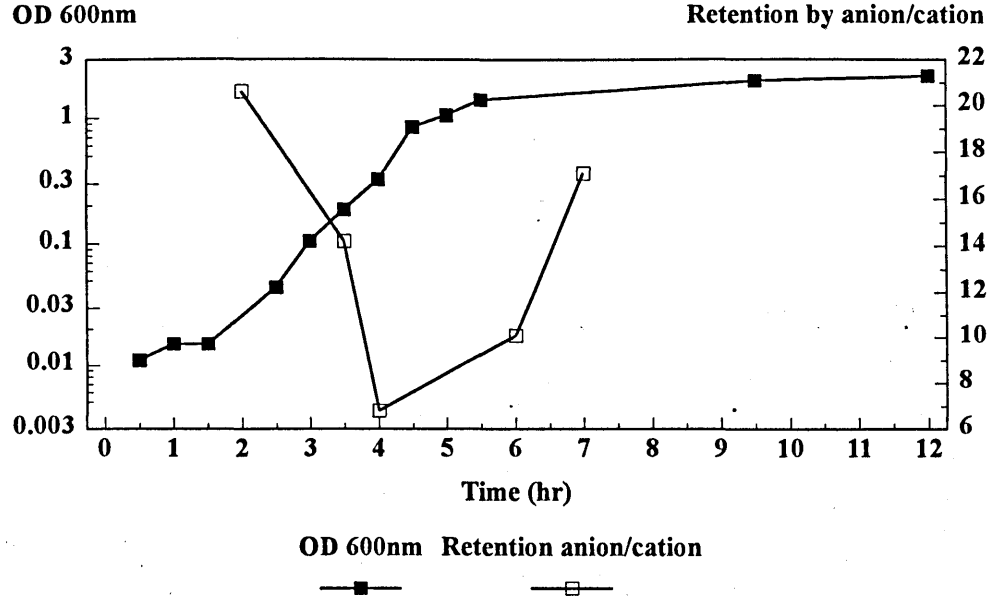
Figure 7.8

Effect of growth phase on (a) hydrophobicity, (b) charge, (c) attachment and (d) cell volume of *S.liquefaciens*

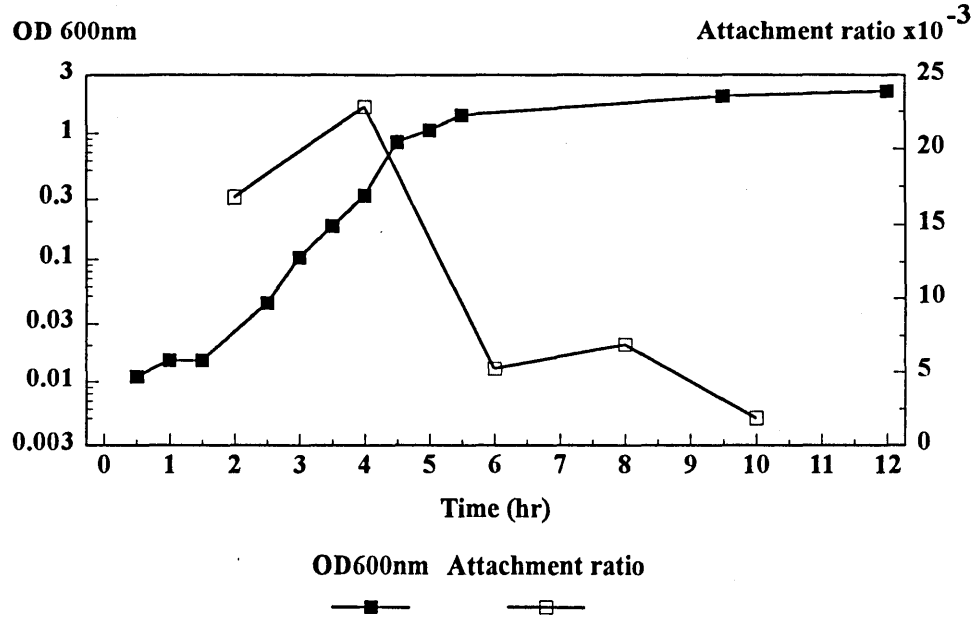
(a) Hydrophobicity



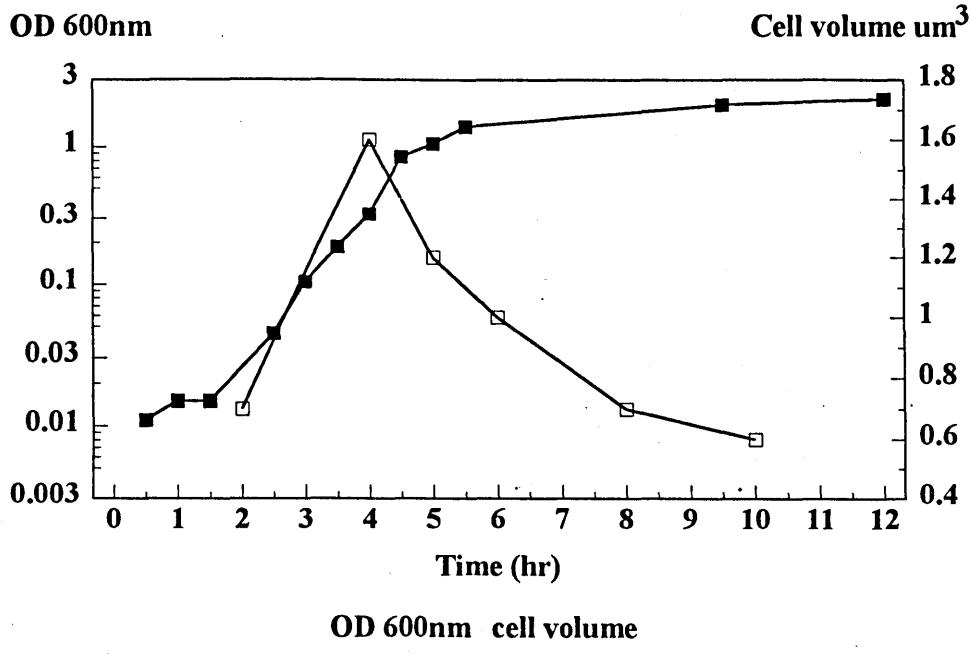
(b) Charge



(c) Attachment



(d) Cell volume



this could be a dispersal mechanism to allow the newly formed daughter cells to be released to colonize new surfaces. The decrease in the hydrophobicity observed during the exponential phase of growth may be due to an increased proportion of newly divided daughter cells. This theory was investigated by determining the mean cell volume of *S.liquefaciens* before and after passage through a phenylsepharose column. Figure 7.8 (d) shows that the mean cell volume was maximal after four hours, and therefore a four hour culture was used for the cell volume studies before and after contact with the sepharose as this contained the highest proportion of elongated cells and therefore presumably newly forming daughter cells.

Table 7.2 shows the mean cell volumes before and after passage through phenyl sepharose and as a control, unsubstituted sepharose. The results show that there was no mechanical entrapment in the matrix of the gel as there was no significant difference in the mean cell volume before and after contact with sepharose. The data also shows that there was a significant reduction in the mean cell volume after contact with the hydrophobic phenylsepharose. If newly formed daughter cells are in fact more hydrophilic than older and elongating cells, then the larger cells will adsorb to the phenylsepharose thus reducing the mean cell volume of the eluate. This would explain the volumes observed and confirm the theory that the hydrophobicity of the cell is related to the cell cycle.

Table 7.2

Effect of passage through a column of phenylsepharose on the mean cell volume of *S.liquefaciens.*

Treatment	Mean cell volume μm^3(sd)
Before	1.6 (0.1)
After phenylsepharose	0.9 (0.2)
After control sepharose	1.5 (0.2)

An exponential phase culture was used so that the mean cell volume was maximal. The mean cell volumes are of five samples and the standard deviations are shown in brackets.

7.8 Conclusions

The examination of cell surface hydrophobicity and charge showed that these surface characteristics vary with the growth conditions, and the effect varies between organisms. Generally, *S.cohnii* was the most hydrophobic organism, *S.liquefaciens* was the most hydrophilic organisms and *P.fragi* had intermediate hydrophobicity.

The enzyme studies indicated that cell surface proteins were involved in the cell surface hydrophobicity of *S.cohnii*, however the changes detected in the cell surface proteins of *P.fragi* did not correlate to changes in hydrophobicity. Similarly, trypsin treatment did not significantly affect the hydrophobicity of *S.liquefaciens.*

Hydrophobicity and charge were found to vary throughout the growth phase. *S.cohnii* and *P.fragi* showed similar trends with respect to hydrophobicity, charge and attachment properties. Hydrophobicity decreased during the exponential phase and was associated with increased cell surface charge. In both *S.cohnii* and *P.fragi* attachment levels were at the

lowest level when hydrophobicity minimal. In contrast, the cell surface charge and attachment of *S.liquefaciens* increased during the exponential phase.

Examination of the attachment data in the light of the hydrophobicity and charge results indicated that the attachment of *S.cohnii* and *P.fragi* was primarily dominated by cell surface hydrophobicity, whilst the attachment of *S.liquefaciens* appeared to be mediated mainly by charge properties.

The change in hydrophobicity through the growth curve suggested that the hydrophobicity of the cell varied through the cell cycle. Examination of the cell volume before and after contact with the hydrophobic phase showed that the mean cell volume of the eluate was significantly lower suggesting that the larger cells had adhered to the hydrophobic phase, and consequently resulted in a reduced mean cell volume in the eluate.

CHAPTER EIGHT

8. GENERAL CONCLUSIONS

There are three sources of contamination in the food processing environment; surfaces, air and people. Bacteria attached to surfaces may therefore be a source of pathogenic and / or spoilage micro-organisms. It is important to understand the attachment of bacteria to surfaces in order to take measures to limit this development. The work presented in this thesis investigated the attachment of three species of bacteria isolated from the food production environment, and modelled the biofilm developed by these three species. Preliminary studies of factory environment biofilms showed that these films ranged from single species biofilms to complex consortia of twelve species and the tri-species biofilm was selected for further investigation because it was a relatively simple mixture that represented both Gram positive and Gram negative organisms.

Modelling of mixed culture biofilm development gave several interesting results. The biofilm that developed on the stainless steel surfaces was relatively heterogeneous in terms of the distribution of the cells across the surface. The biofilm appeared to be composed of microcolonies that could be several cells deep with extensive exopolymer material present, although in certain areas the substratum was visible. The major control of the surface population in the food processing environment is the cleaning and disinfection regime, and this is timed to coincide with production windows. Production lines may run for as little as an hour to several days and therefore the time available for biofilm development may be relatively short. Consequently the biofilms developed in this study are comparable to the biofilms that develop in the factory environment (Holah and Kearney, 1992).

The growth media and the growth phase influenced attachment. Generally, *S.cohnii* had the highest attachment ratio, *S.liquefaciens* the lowest and *P.fragi* showed intermediate levels of attachment. Similar attachment properties were observed from a mixed culture of the three organisms. These results demonstrate that the environmental conditions significantly affect the levels of attachment and that the attachment potential of each organism was similar in the presence of the other species.

Examination of the reasons for the differences in attachment abilities of the three organisms involved investigation of outer membrane proteins, lipopolysaccharides, exopolysaccharide production, hydrophobicity and charge.

There was no correlation between either the outer membrane protein or lipopolysaccharide expression and the attachment characteristics, although both outer membrane proteins and lipopolysaccharide were subject to considerable variation in response to the environmental conditions.

The hydrophobicity and charge studies showed several interesting correlations to the attachment levels observed. The attachment of *S.cohnii* and *P.fragi* correlated with cell surface hydrophobicity, whilst the attachment of *S.liquefaciens* was more dependant upon cell surface charge. This data confirmed Van Loosdrecht's observations that the attachment of hydrophobic organisms is generally mediated by hydrophobic interactions whilst the attachment of hydrophilic bacteria is primarily controlled by charge properties (Van Loosdrecht *et al.*, 1987). Understanding the mechanisms of bacterial attachment to surfaces may allow the development of approaches to limit this attachment, for example surface treatments or coatings may be used to modify surface charge and hydrophobicity and therefore attachment. However it is difficult to envisage how this would be achieved in practice as the surface would rapidly become conditioned by adsorbing macromolecules which would mask any changes.

The cell surface hydrophobicity and charge varied with growth conditions and growth phase and in the case of *S.cohnii* appeared to be attributable to surface proteins. The hydrophobicity data also demonstrated the existence of sub-populations within a culture that related to cell cycle events and was characterized by the change in cell volume and cell surface hydrophobicity. These results may have relevance for the dispersal of bacteria from surfaces. Gilbert *et al.* (1991) found that daughter cells released from a perfused filter system were more hydrophilic and therefore readily dispersed in the aqueous phase.

There are several different factors that may play a role in attachment (hydrophobicity, charge, EPS production, OMP expression and LPS expression) and it is difficult to explain attachment in terms of any single factor. Statistical tools such as Taguchi Exploratory

Experimental Design could have been used for the analysis of complex situations such as this where there are several critical factors involved. However these systems do not allow for any interaction between factors.

The data presented in this thesis also demonstrated some interesting characteristics of surface attached bacteria. Attached bacteria were shown to be more active than liquid phase cells, possibly due to concentration of nutrients at the solid-liquid interface. Attached bacteria were also found to produce significantly higher levels of exopolysaccharide material. The role of polysaccharides in attachment and biofilm development is dependent upon the microorganisms involved, however exopolysaccharides are generally thought to be more important in the subsequent biofilm development rather than the initial attachment process. The data presented in this study illustrated that exopolysaccharide was found in higher levels around surface attached organisms, and as exopolysaccharide production is energetically expensive to the cell this increased production must be of ecological advantage to the cell, in this case promoting biofilm development. The presence of exopolysaccharide material may protect cells from disinfection and desiccation and therefore allow organisms to remain as a source of contamination.

Bacteria attached to surfaces will continue to be a cause for concern in the food processing environment particularly with the trends towards longer process running times and increased production of chilled foods which require high standards of hygiene.

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9. REFERENCES

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